Destabilization of the NMJ by Neurotrypsin-Mediated Agrin Cleavage Results in Precocious Sarcopenia

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CONTENTS

Abstract

Sarcopenia, the progressive decline in skeletal muscle mass and strength that occurs with aging, contributes substantially to impaired mobility of the elderly and has been associated with an increased risk of morbidity, disability, and mortality. Numerous age-associated processes have been shown to influence muscle mass and function, but the pathogenic mechanism leading to sarcopenia is still poorly understood. We found that destabilization of the neuromuscular junction (NMJ) via overexpression of the agrin-cleaving protease neurotrypsin in motoneurons installed the full sarcopenia phenotype in juvenile and young adult mice. Characteristic alterations included a reduced number of muscle fibers, increased heterogeneity of fiber thickness, angulated fibers, centralized nuclei, fiber-type grouping, and an increased proportion of type I fibers. We propose the neurotrypsin-overexpressing mouse as an animal model for sarcopenia, providing a well-defined aging-like neuromuscular environment in an otherwise healthy animal. The induction of sarcopenia at young age challenges several hypotheses about its etiology. Age-related endocrine changes, physical inactivity, inappropriate food intake, mtitochondrial dysfunction due to life-long accumulation of oxidative damages, and changes in muscle protein synthesis appear to be dispensable for the development of sarcopenia. Similarly, the loss of motoneurons, generally thought to be the main sarcopenia-promoting process, may not be a necessary condition for sarcopenia, since a reduced number of motoneurons was not found in neurotrypsin-overexpression animals. Our results rather suggest NMJ destabilization and fiber denervation at the onset of a final common pathway leading to sarcopenia. As in conditions with enhanced agrin cleavage, severe fragmentation of the NMJs that eventually lead to the dispersal of the postsynaptic receptor clusters was observed with aging. Whether neurotrypsin or agrin cleavage are responsible for age-related NMJ destabilization and sarcopenia could not be fully elucidated. Neurotrypsin-dependent agrin cleavage is ruled out as a single suspect, as absence of neurotrypsin activity does not protect mice from developing sarcopenia at old age. Overexpression of a neurotrypsin-resistant form of agrin prevented sarcopenia in young neurotrypsin-overexpressing mice, but failed to rescue these mice from developing sarcopenia at old age. However, small beneficial effects of transgenic agrin on the morphology of aged muscles provide some evidence that the amount of full-length agrin may influence the development of sarcopenia. Whether this influence is related to agrin cleavage remains unclear. Other processes that affect the stability of the NMJ may adopt a similar pathway on the way to NMJ destabilization, muscle fiber denervation, and sarcopenia.

Zusammenfassung

Sarkopenie bezeichnet den kontinuierlichen Muskelschwund mit fortschreitendem Alter, der sowohl durch den Verlust an Masse, wie auch an Kraft der Skelettmuskulatur charakterisiert ist. Sarkopenie trägt entscheidend zu eingeschränkter Mobilität unter älteren Personen bei und wird mit erhöhter Morbidität, Behinderung und Sterblichkeit in Verbindung gebracht. Weil diverse altersbedingte Prozesse einen Einfluss auf den Verlauf der Sarkopenie haben können, sind die entscheidenden Mechanismen die den Muskelschwund auslösen nach wie vor ungeklärt. In dieser Arbeit wird gezeigt, dass die Destabilisierung der neuromuskulären Endplatte, induziert durch Überexpression der Agrin-spaltenden Protease Neurotrypsin, Sarkopenie in jungen Mäusen verursacht. Charakteristische morphologische Veränderungen der Skelettmuskulatur sind eine reduzierte Anzahl Muskelfasern, erhöhte Heterogenität der Faserdicke, abnormal geformte Muskelfasern, zentrierte Kerne, Gruppierung der Fasertypen und ein erhöhter Anteil an Typ-I-Fasern. Die Neurotrypsin-überexprimierende Maus ist ein Tiermodell für Sarkopenie mit einem gealterten neuromuskulären System in einem ansonsten gesunden Organismus. Die Induktion von Sarkopenie in jungen Mäusen stellt diverse Prozesse, die bisher als Sarkopenie-auslösend bezeichnet worden sind, in Frage. Altersbedingte hormonelle Veränderungen, physische Inaktivität, mangelhafte Ernährung, Versagen der Mitochondrien durch lebenslange Ansammlung von oxidativen Schäden und Veränderungen der Synthese von Muskelproteinen scheinen keine essentiellen Voraussetzungen für die Entwicklung von Sarkopenie zu sein. Das gleiche gilt für den Verlust von Motoneuronen, der laut vielen Berichten entscheidend zum altersbedingten Muskelschwund beiträgt, da kein solcher Verlust in jungen Mäusen mit Sarkopenie gefunden wurde. Der Sarkopenie auslösende Prozess scheint eher die Destabilisierung der motorischen Endplatte und die Denervierung von Muskelfasern zu beinhalten. Sowohl in Neurotrypsin-überexprimierenden, wie auch in alten Mäusen wurde eine Fragmentierung der neuromuskulären Endplatten gefunden, die mit dem vollständigen Verlust des Kontakts zwischen Nerv und Muskel enden kann. Daher könnten Endplatten-stabilisierende (Agrin), wie auch -destabilisierende (Neurotrypsin) Proteine eine wichtige Rolle in der Entstehung von altersabhängiger Sarkopenie spielen. Experimente mit alten Neurotrypsin-defizienten Mäusen haben aber gezeigt, dass Sarkopenie auch in Abwesenheit der Agrin-spaltenden Protease vorkommen kann. Die Überexpression einer Neurotrypsin-resistenten Agrin Form konnte zwar Sarkopenie in jungen Neurotrypsin-überexprimierenden Mäusen verhindern, nicht aber die Entwicklung der altersabhängigen Sarkopenie. Transgenes Agrin beinflusste allerdings die Morphologie von alten Muskeln positiv, was auf einen Abhängigkeit des Verlaufs der Sarkopenie von der Menge an ungeschnittenem Agrin deuten könnte. Zusammenfassend bleibt unklar ob die Prozessierung von Agrin an der Induktion von altersabhängiger Sarkopenie beteiligt ist. Es ist denkbar, dass andere Prozesse, die zur Destabilisierung der motorischen Endplatte führen, auf die gleiche Weise Sarkopenie verursachen.

1 INTRODUCTION

1.1 The neuromuscular junction (NMJ)

The vertebrate skeletal neuromuscular junction (NMJ) is the best studied of all synapses, due to his size, accessibility and simplicity[1,2]. It was used as model system for the study of synaptic transmission, development and modifications. The first demonstration of synaptic transmission was for example accomplished with the NMJ[3]. Later, the nicotinic acetylcholine receptor (AChR) was the first neurotransmitter receptor to be purified and cloned[4]. Studies of the neuromuscular development have spanned a century, with progress determined in large part by the tools available. Light microscopic methods were used to elucidate some of the main features of neuromuscular synaptogenesis and regenerations. Techniques of intracellular recordings and electron microscopy resulted in a sophisticated view of its structure and function. Later, it was realized that synapse formation involves elaborate signaling between nerve and muscle[5]. Molecular biological techniques permitted isolation of candidate signaling molecules and demonstration of their bioactivities *in vitro*[6]. In the last two decades, technical advantages in imaging and molecular genetics have permitted the *in vivo* confirmation of specific hypotheses derived from earlier work.
In this study, we report that destabilization of the NMJs, via enhanced cleavage of the synapse stabilizer agrin, leads to severe degeneration of skeletal muscles. This muscle degeneration shares numerous characteristics with the age-related loss of skeletal muscle mass and function known as sarcopenia. The stability of the NMJ and hence, NMJ protecting or disrupting agents, may be essential factors influencing the integrity of the neuromuscular system. We propose that sarcopenia results from the deterioration of this system and involves the dispersal of the NMJ that is followed by the degeneration of the skeletal muscle.

1.1.1 Structure

The NMJ consists of three cellular components, motoneuron, muscle fiber and Schwann cell[7]. The synaptic portions of all three are highly specialized, containing high concentrations of organelles and molecules found at low concentrations extrasynaptically. The motor nerve terminal is specialized for neurotransmitter release, bearing a large number of synaptic vesicles containing the neurotransmitter acetylcholine (ACh). Many of the vesicles are focused at dense patches on the presynaptic membrane, called active zones, at which the vesicles fuse with the membrane and release their contents into the synaptic cleft. The postsynaptic membrane is specialized to respond rapidly and reliably to neurotransmitter released from the overlying nerve terminal. It bears an extremely high concentration of AChRs[8], and other signaling molecules[9-11]. The postsynaptic membrane is organized in junctional folds that open directly opposite the active zones. AChRs are concentrated at the crests, sodium channels at the depths of folds[12]. Schwann-cell processes cap the nerve terminal and insulate it from the environment. Finally, a basal lamina ensheats each muscle fiber, passes through the synaptic cleft and extends into the junctional folds. The major components are collagen IV, laminin, entactin, and heparan sulfate proteoglycans. The synaptic basal lamina also contains agrin, an important signaling molecule.

1.1.2 Formation

The formation of the NMJ starts with the establishment of rudimentary postsynaptic specializations

in the muscle membrane, referred to as prepatterning. A spatially restricted pattern of myonuclear AChR expression and the corresponding formation of surface AChR clusters determine the region of the prospective endplates without neuronal influence[13-15]. The prepatterning does not depend on agrin but on the function of MuSK, the muscle-specific kinase. Recently, it has been shown that these prepatterned clusters can develop to elaborately branched structures without neural influence[16]. These aneural pretzel-like structures share a number of characteristics with the mature postsynaptic apparatus, such as colocalization of several postsynaptic markers, clustering of subjacent myonuclei, and dependence on MuSK and rapsyn for their formation. They develop through a series of steps mirroring those seen *in vivo*. However, formation of nerve-muscle contacts, dispersal of ectopic postsynaptic structures, synapse maintenance, intramuscular nerve branching, and neuronal survival requires reciprocal signals from nerve and muscle. The arriving nerves orchestrate coexisting synapse-stabilizing and synapse-dissolving mechanims to generate a well balanced match between pre- and postsynaptic elements. Thereby, prepatterned AChR clusters may be recognized by the ingrowing axons and incorporated into synapses[17]. Nerve-derived agrin is released from the presynaptic motoneuron and subsequentially stabilizes postsynaptic specialization. Agrin is a heperan sulphate proteoglycan of about 400-600 kDa[18,19] that was initially purified as an activity that induces enhanced clustering of AChRs on myotubes *in vitro*[20,21]. It is synthesized by motoneurons, released from nerve terminals and incorporated into the basal lamina of the synaptic cleft[21,22]. Gain- and loss-of-function studies indicated that agrin is a critical nerve-derived organizer of postsynaptic differentiation. The overexpression of agrin in the absence of nerves led to the formation of remarkably complete postsynaptic apparatus on muscle fibers[23-25]. Conversely, postsynaptic differentiation was impaired in agrin-deficient mice. The overall levels of AChR were normal, but few nerve-muscle contacts were accompanied by detectable AChR clusters or other postsynaptic specializations[26]. Thus, agrin is necessary and sufficient for postsynaptic differentiation. An important discovery was that nerve-, but not muscle derived agrin is capable to induce postsynaptic differentiation. Only neurons express the so called z/B$^+$ agrin isoform, which results from alternative splicing and posseses an amino acid insert at the z/B site[27-30] (see below). This agrin form is 1000-fold better at inducing AChR clusters compared to the isoform expressed by muscle, and is essential for synaptic development[31,32].

1.1.3 Agrin signalling

Several molecules on the myotube surface are capable of interacting with agrin, including dystroglycan, integrins, N-CAM, and laminins[33-39]. However, none of these essentially contribute to postsynaptic differentiation *in vivo*. In contrast, a transmembrane protein tyrosine kinase, MuSK, has emerged as the agrin receptor, even though it does not directly bind agrin[40]. MuSK is selectively expressed by skeletal muscle, where it co-localizes with AChRs in the postsynaptic membrane[10]. MuSK defcient mice displayed the same neuromuscular defects than the agrin deficient mutants[41]. Myoutubes cultered from MuSK deficient mice fail to form AChR clusters, either spontaniously or in response to agrin, and this defect can be rescued by reintroduction of MuSK. Furthermore, the application of agrin to myotubes results in a rapid phosphorylation of MuSK[42]. However, a direct interaction of agrin and MuSK was never demonstrated. Recently, LRP4, a member of the low density lippotein receptor family, has been reported to serve as coreceptor for agrin, forming a complex together with MuSK in a manner that is stimulated by agrin[43,44]. Downstream of MuSK, a 43 kDa cytoplasmic protein called rapsyn (receptor-associated protein at the synapse) is a critical effector of AChR clustering. AChRs and rapsyn are precisely colocalized at the NMJ as soon as clusters form and may interact directly[45,46]. No AChR clusters form in muscles of rapsyn-deficient mice, or in cultured myotubes from the mutants, even following treatment with

agrin[47]. However, MuSK is still clustered at synaptic sites. Therefore, rapsyn is necessary for AChR clustering and a downstream target of MuSK. Agrin, via MuSK, may increase the amount of rapsyn that associates with AChRs in the muscle membrane to levels that enhance clustering during synapse formation and stabilization. The association with rapsyn stabilizes the AChRs in the cell membrane by slowing their metabolic degradation. Rapsyn also mediates the attachment of AChRs to the utrophin-associated complex required for AChR stabilization and maturation of the NMJ[48]. Proteins within the utrophin-associated complex such as α-dystrobrevin and α-syntrophin are also important for signaling events that affect NMJ stability and function. The mechanism by which activation of MuSK induces rapsyn and AChR clustering is not yet fully understood but may involve the non-receptor tyrosin kinases Abl1 and Abl2[49,50]. In mouse muscles, Abl kinases are located at the postsynaptic membrane of the developing NMJ. Their activity is required for agrin-induced AChR aggregation and enhancement of MuSK tyrosine phosphorylation. MuSK and Abl kinases effect reciprocal tyrosine phosphorylation and form a complex after agrin engagement. Abl kinases appear to provide the developing NMJ with the kinase activity that is necessary for signal amplification. MuSK activation not only enhances AChR clustering but also the synaptic concentration of ErbB receptors, which transduce signals from neuregulin. Neuregulin is a synaptic proteoglycan implicated in AChR synthesis in subsynaptic nuclei[51,52]. Synapse-specific gene transcription contributes essentially to the differentiation and stabilization of the postsynaptic membrane. However, recently, erbB2 and erbB4 have been conditionally deleted from skeletal muscle, rendering muscle fibers insensitive to neuregulin, but preserving neuregulin signalling to Schwann cells[53]. The resulting animals were viable and did not suffer from neuromuscular deficits. Structure and function of the NMJs was largely normal, despite a small decrease in AChR density. Surprisingly, synapse-specific transcription was maintained with only a 20-30% reduction in synaptic AChR transcripts. Thus, neuregulin signaling to muscle is not required for at least the principal aspects of postsynaptic development. Instead, defects observed in the absence of neuregulin-ErbB signaling are probalbly secondary to aberrant Schwann cell development[17]. Although transcriptional specialization of synaptic nuclei can occur in the absence of innervation[13,14], its maintanance appears to require agrin and MuSK[26,41,47,53]. The mechanism by which agrin supports synapse-specific gene transcription remains to be elucidated, but it does not include autocrine neuregulin-ErbB signaling[32]. Anchoring of synaptic nuclei to the NMJ appears to depend on Syne-1. Syne-1 is a large protein with an N-terminal actin-binding domain, spectrin repeats, and a C-terminal domain that mediates association with the nuclear envelope. It interacts with MuSK and is concentrated in the envelope of synaptic nuclei at the NMJ[54]. The transgenic expression of a dominant-negative Syne-1 fragment prevented the accumulation of subsynaptic nuclei, suggesting that Syne-1 is required for anchoring of synaptic nuclei, perhaps through interaction with MuSK[55]. However, despite the absence of subsynaptic nulcei, there were no apparent NMJ defects. Thus, subsynaptic nuclei are dispensable for formation and topological maturation of the NMJ, although transcriptional specialization of nuclei in the vicinity of the synapses might be required. Extrasynaptically, the AChR synthesis is repressed by electrical activity[56]. Thus, AChRs are negatively regulated by their ligand in extrasynaptic regions, but immune for the repressive effects of activity at the synapse (**Fig. 1**).

1.1.4 Dispersal of unproteced AChR

A similar mechanism is responsible for the dispersal of AChR clusters that are not opposed by nerve endings and, thus, not supported by agrin (**Fig. 1**). ACh negatively regulates neuromuscular synapse formation by inducing the dispersal of AChR clusters that have not been stabilized by nerve-derived agrin[57,58]. ACh-mediated dispersion of AChRs may occur by endocytosis causing the loss of AChRs and by a redistribution of AChRs in the plane of the membrane. There is

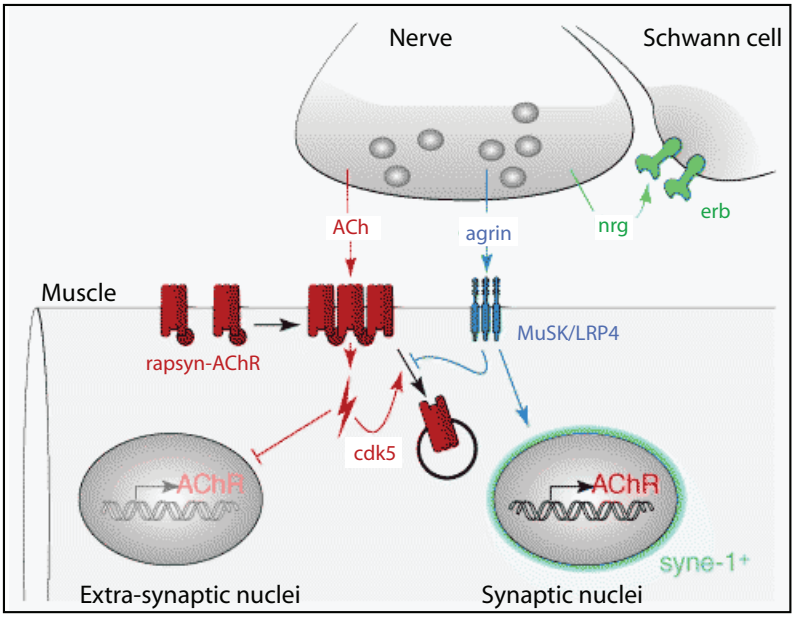


Figure 1 A molecular model of postsynaptic development at the NMJ. ACh-induced action potentials (red flash) disperse AChR clusters via endocytosis and repress extra-synaptic transcription of AChR and other synapse-specific mRNAs. Neuronally released agrin (blue) interacts with MuSK via LRP4 to stimulate co-clustering of AChRs and rapsyn (red), probably by repressing neurotransmission-induced loss of AChRs. Synaptic transcriptional specialization contributes to synaptic AChR accumulation and requires agrin and MuSK. Neuregulin signaling primarily affects muscle indirectly via its effects on perisynaptic Schwann cells and may play a modulatory role in this process. Syne-1 (cyan) is concentrated in the envelopes of synaptic nuclei at the NMJ and may be required for anchoring of synaptic nuclei, perhaps through interaction with MuSK. From Kummer et al.[17]

evidence for ACh to signal via cyclin-dependent kinase (cdk)-5-dependent mechanism that could destablize AChR directly or impair assembly and recycling of AChR clusters. Consistent with an ACh-induced dispersal mechanism, denervated endplates develop normally, initiate fold formation on schedule and maintain their accumulation of AChRs, acetylcholine esterase (AChE) and synaptic nuclei[59]. Moreover, AChR aggregates persists longer in aneural muscles than in innervated muscles of agrin mutants[13,14]. Finally, double-knockout mice, deficient in both agrin and choline acetyl transferase, the sole synthetic enzyme for ACh, were generated[57,58]. In the mutant mice NMJs form, undergo pre- and postsynpatic differentiation, and persist until birth when the animals die. Absence of both dispersal (ACh) and protection (agrin) mechanism results in persistance of the NMJs. The role of agrin *in vivo* appears more to relate to synapse maintenance than synapse formation. Agrin acts as a protector, preventing the AChR clusters from disappearance by counteracting a local dispersal effect of neurotransmission at synaptic sites. This mechanism garantuees the perfect alignment of pre- and postsynaptic sites. Nerve-derived agrin specifies the innervated postsynaptic specializations as those to be maintained and further differentiated, whereas nerve-derived acetylcholine-dependent signals to the muscle fiber shut off AChR expression outside of the synaptic area and trigger the “dispersal mechanism” for non-innervated and, thus, unprotected postsynaptic specializations. As a consequence, postsynaptic specialization can be remarkebly stable in the absence of the nerve, since then, not only protective but also destructive signals are absent.

1.1.5 Presynaptic differentiation

The agrin-mediated differentiation of the postsynaptic apparatus initiates reciprocal signals to the presynaptic nerve, resulting in the cessation of motor axon growth and the maturation of the presynaptic terminal[60]. The phenotype of agrin and MuSK deficient mice provide strong evidence that postsynaptic differentiation is necessary for subsequent induction of presynaptic development. The motoneuron terminals fail to differentiate, remaining highly dynamic and extending processes along the muslce surface[26,41]. Further evidence that postsynaptic differentiation is necessary for the subsequent induction of the presynaptic nerve terminal comes from muscle

transplantation studies in which MuSK or rapsyin deficient muscles are transplanted into wild type animals[60]. Nerve terminals contacting transplanted MuSK or rapsyin deficient muscles remained undifferentiated and were observed to remodel continously over the course of several months. Initial steps in presynaptic differentiation occurred but wild type nerve terminals were unable to mature when postsynaptic differentiation was blocked on MuSK or rapsyin deficient muscles. Thus, the presynaptic maturation crucially depends on postsynaptic differentiation. The nature of the muscle-derived signal that is necessary to induce presynaptic maturation has not been clealy defined. However, it may involve signaling via laminins in the synaptic basal lamina, which is necessary for several aspects of presynaptic development[61-63]. Key signaling molecules that induce presynaptic differentiation may be deposited in the synaptic basal lamina by the muscle following the activation of MuSK.

1.1.6 Synaptic maturation

During the weeks after initial nerve-muscle contact, the plaque-like early NMJ transforms to the typical pretzel-like structure of the mature endplate[1,32,64]. The plaque perforates as AChRs are lost from regions within it. As this process continous, the plaque transforms into a series of branches, giving the junction the pretzel-like appearance. The topography of the postsynaptic membrane changes from an initially flat and apparently homogenous area to a complicated invaginated, pretzel-like structure with a compartmentalized molecular composition. During the same period, all but one of the initially multiple nerve terminals projecting on one postsynaptic specialization withdraw in a process called synapse elimination[65]. Polyneuronal innervation originates by the convergence of multiple motor axons on individual muscle fibers. Retraction of some terminal branches from each muscle fiber leads to the loss of multiple innervation without any changes in the total number of motoneurons innervating the muscle[66,67]. Thus, the size, but not the number of motor units decreases during synapse elimination. The elimination process include a stepwise loss of terminal branches, which undergo a program of atrophy, detachment, and withdrawal. Eventually, the axon forms a retraction bulb and withdraws from the endplate[68,69]. Serial electron microscopy revealed an unusual cellular mechanism that removes axons at the NMJ[70]. Retracting motor axons shed numerous membrane bound remnants (axosomes) that contain a high density of synaptic organelles and are formed by engulfment of axon tips by Schwann cells. In most of the cases, the remaining nerve then extend axonal processes to takeover the vacated postsynaptic sites. When the remaining axon does not reoccupy a site, the postsynaptic receptors rapidly disappear, most likely due to active, Ach-induced dispersal. The synapse elimination is competitive and activity dependent[71], and its dynamics has been analyzed *in vivo*[72] (**Fig. 2**). Very early it has been recognized that activity is involved in synapse elimination, since blockage of activity prevented elimination[73,74]. Moreover, the relative synaptic efficiacy of two competing axons at a single NMJ influences the outcome of synaptic competition. This has been demonstrated by genetically reducing synaptic efficiacy at one of two axons innervating a target[75]. This was achieved by reducing neurotransmission in a subset of motoneurons by conditional genetic deletion of choline acetly transferase. More active inputs were strongly favoured competitors during synapse elimination. There are several lines of evidence indicating that the synaptic competition is mediated through the muscle cell. Focal blockage of receptor activation within a small region of the NMJ induced for example elimination at these sites, indicating that local differences in receptor activation can drive elimination[76]. Postsynaptic activity-dependent mechanisms appears to function locally to drive the elimination of neighboring inputs. Consistent with this idea, two motoneuron inputs can be maintained on the muscle surface if they are seperated by enough distance[77]. Mainly two models have been formulated to explain activity-dependent competition

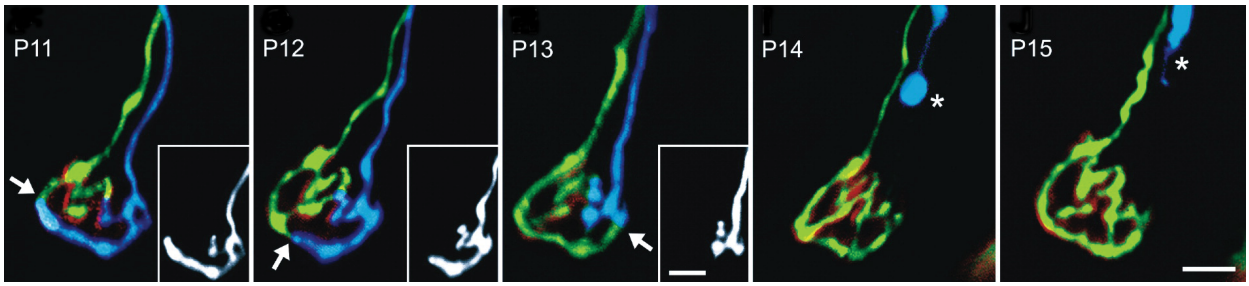


Figure 2 *In vivo* observation of synapse elimination. The same multiply innervated junction is imaged in neonates between postnatal day 11 (P11) and 15 (P15). Although in this case the subset CFP axon (blue and insets) has greater terminal area (70%) at the first view, it progressively withdraws from the junction (arrows and asterisks). Insets, 70% size reduction. Scale bars, 10 µm. From Walsh and Lichtman.[72]

leading to input elimination at the NMJ[1]. In the first model, presynaptic inputs compet for access to limiting amounts of muscle-derived trophic signals. Less avtive inputs receive less trophic support and are subsequentially eliminated. A second model involves signalling mechanisms that actively drive the process of elimination of less active inputs. These putative negative signals have been termed synaptotoxins or punishment signals. Since activity-dependent competition appears to be mediated through the muscle, this model also includes the idea that less active inputs are more susceptible to synaptotoxins or that more active inputs are protected, or both. However, recently it has been observed that an input could initially begin the process of elimination and then subsequentially reverse this process by growing to become the single input that is maintained[72]. Thus, the mechanisms underlying input elimination are reversible and the elimination process appears not to progress monotonically but to be continually driven until an entire input is eliminated. Moreover, an initially ineffective input can overtake an initially more effective input, indicating that the competitive vigor can change dramatically over the time course of the synaptic competition. Thus, there must be mechanisms in addition to receptor activation that determine the outcome of competition-driven input elimination[2]. In vivo examination of synaptic competition between two motoneurons at each muscle fiber where they converge, led to further insights into synaptic competition[78]. First, one motoneuron loses every competition with the second, suggesting that one motoneuron has an intrinsic competitive advantage over the other neuron throughout its entire presynaptic arborization. If synaptic efficiacy can be linked to axonal activity, the differences in motoneuron activity could be one mean to bias competition throughout the entire arborization of a single motoneuron. Second, motoneurons with larger total arboriazation are at disadvantage in competitions against motoneurons with smaller total arborization. This observation has been explained by a cell-wide finite resource that influences the competitive vigor and becomes diluted as a motoneuron gains territories. This finite resource could be neurotransmitter since the depletion of ACh by genetic deletion of choline acetyl transferase leads to a competitive disadvantage[75]. Such growth restriction may act to prevent excessive expansion of a single arborization. Interestingly, the largest motor units are generally those that are the least active; they are recruited last during movements[79,80]. Motoneurons with larger arborizations may be less active and hence, loss more synaptic targets as a result of synaptic competition than smaller, more active motor units. After a motoneuronal input has been established, the corresponding muscle fiber becomes unresponsive towards further innervation. A foreign nerve, implanted on a muscle, cannot induce the formation of new NMJs as long as the muscle is innervated[77].

1.1.7 Synapse maintenance

Mature NMJs persists for the life of an animal. Time lapse imaging in mice shows that most NMJs

are remarkebly stable, they do not add or lose branches even though they enlarge considerably as the animal grows[81-83]. *In vivo* visualization of the growth of pre- and postsynaptic elements showed that the NMJ primarely grow by expansion of existing motor nerve terminal and postsynaptic receptor regions without the addition or loss of synaptic area[82,83]. Each NMJ grew at the same rate that muscle fiber enlarged, suggesting that junctional growth might be a mechanical consequence of muscle fiber growth. During this process, the number of AChRs at the synapses increased, but AChR density remained constant. Since the alignment between growing motor nerve terminal and postsynaptic regions is maintained, nerve terminal growth may be a consequence of its adhesion to growing postsynaptic specializations. Synaptic turnover appears not to be a necessary feature of synaptic maintenance. Experiments on denervated and damaged muscles showed that postsynaptic specialization persist for long periods in the absence of a nerve[84-86]. Moreover, when myotubes regenerate following injury in the absence of the nerve, postsynaptic specialization accumulate at the original synaptic sites[87-89]. Components of the basal lamina remain at synaptic sites after removel of the nerve and direct the biochemical and structural organization of the subsynaptic membrane. Thus, the synaptic basal lamina, containing agrin, seems to be responsible for the maintenance of the postsynaptic apparatus, regardless if the nerve is present or not. However, the basal lamina is not sufficient for complete maintenance of the nerve terminal. The degeneration of mouse muscle fibers leads to the withdrawal of some terminal branches from endplates within two days[90]. Thus, the nerve terminal may be dependant on an ongoing production of proteins by the muscle fiber[60].

Interestingly, silencing of MuSK expression by RNA interference results in dissassembly of existing NMJs in adult mice[91]. Short hairpin RNA (shRNA) were used to enable prolonged and stable suppression of gene expression *in vivo*. The alterations of the postsynaptic structures after applying MuSK shRNA ranged from fragmentation to severe disassembly of postsynaptic AChR clusters. In response to abrogation of postsynapse integrity, presynaptic nerve endings began to sprout. In certain cases, the entire possynaptic structure disappeared and the motoneuron terminal was the only evidence that an NMJ had been present before. These results indicate that a continuous presence of MuSK is required for the maintainance of the NMJs. Absence of MuSK leads to active dispersal of the postsynpatic sites, most likely due to an ACh-induced mechanism. Furthermore, a compact postsynaptic structure appears to be required to maintain presynaptic integrity.

The maintenance of the junction seems to be impaired in older age. The number of AChRs per junction decreases, extrajunctional recptors appear, and vacated synaptic sites and terminal sprouts become more prevalent in old muscles. Branches retract and the number of discrete regions of AChR density is increased, leading to a fragmented appearance of the junction[82,92-94]. These changes involve both pre- and postsynaptic disassembly and thus resemble synapse elimination rather than denervation[1]. A progressive loss of synaptic contact in individual NMJs and, finally, the loss of entire NMJs with some reinnervation at others has been reported[95].

1.2 Agrin is crucial for the maintenance of the NMJ

Agrin was initially purified using basal lamina extracts from the synapse-rich electric organ of the pacific electric ray *Torpedo californica*[20]. It is a protein of more than 2000 amino acids with a predicted molecular mass of 225 kDa. The extensive N- and O-linked glycosylation of the amino-terminal half increases the apparent molecular mass of agrin to about 600 kDa. At least three of the O-linked carbohydrate attachment sites function as docking sites for heparan sulfate glycosaminoglycan side chains and assign agrin to the family of heparan sulfate proteoglycans[18,96].

Agrin is composed of numerous domains that are homologous to motifs found in other basal lamina proteins (**Fig. 3**). It contains 9 FS (follistatin–like), 2 LE (laminin-EGF-like), one SEA (sperm protein enterokinase and agrin), 4 EGF (epidermal growth factor-like), and 3 LG (laminin

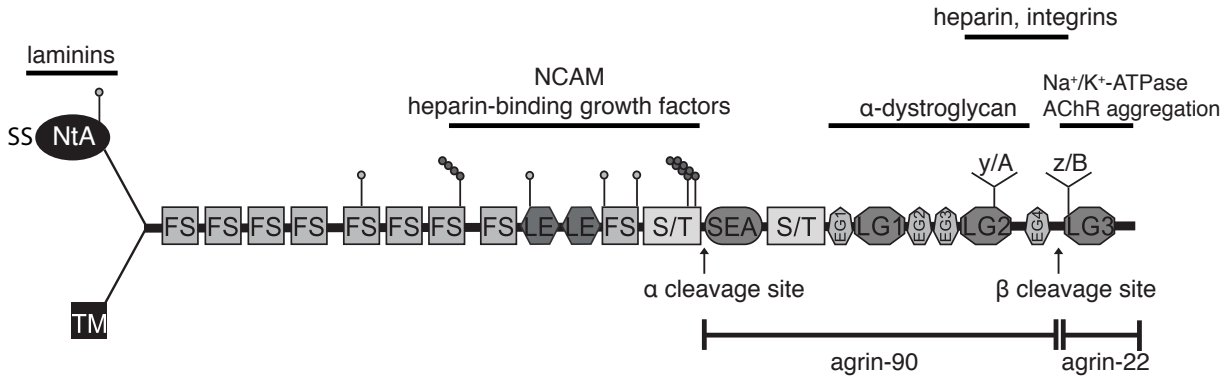


Figure 3 Schematic representation of agrin, showing its structural domains, the binding regions of some of the most important partners and the neurotrypsin-specific cleavage sites. The signal sequence (SS) and amino (N)-terminal agrin domain (NtA) are present in an agrin isoform that is localized to the NMJ. These two regions are responsible for the release of agrin (SS) and the binding to the laminins in basal laminae (NtA). The alternatively spliced type II transmembrane segment (TM) of agrin anchors this isoform at places that are devoid of basal laminae such as the brain. The amino-terminal half of agrin is highly glycosylated at N-linked glycosylation (circles) and glycosaminoglycan (multiple circles) attachment sites. These regions are also involved in binding to neural-cell adhesion molecule (NCAM) and heparin-binding growth factors. The carboxy-terminal, 95-kDa part of agrin (starting with the first epidermal growth factor (EGF)-like domain (EG)) is fully active in AChR aggregation and contains binding sites for α-dystroglycan, heparin, some integrins, and the sodium/potassium-ATPase. Two additional splice sites, y/A and z/B, are located in the C-terminal part of agrin. The most carboxy-terminal, 21-kDa LG domain (LG3) is sufficient to induce AChR aggregation at low potency. Agrin can be proteolytically processed by neurotrypsin at the α- and β- cleavage sites, generating a C-terminal 22 kDa fragment (agrin-22) and a 90 kDa middle fragment (agrin-90). FS, follistatin-like domain; LE, laminin EGF-like domain; S/T, serine/threonine-rich region; LG, laminin globular domain; SEA, sperm protein, enterokinase and agrin domain.

globular) domains. Agrin exists in several isoforms that are generated by alternative splicing[96]. The N-terminus can include a laminin-binding domain or a transmembrane region, which are constituents of a secreted or a type II transmembrane form of the protein, respectively. The later isoform is predominantly expressed in the brain, whereas the first is expressed by motoneurons. It is transported down the motor nerve and released from the nerve ending into the synaptic cleft of the NMJ, where it becomes an essential component of the synaptic basal lamina and interacts with receptors on the postsynaptic membrane to stabilize postsynaptic membrane specialization formation[97]. The C-terminal moiety of agrin comprises two additional splice sites, called y/A and z/B (A and B and y and z are used to denote these sites in chicks and mammels, respectively). Splicing at these sites gives rise to protein variants that can contain 0 or 4 amino acids at the y/A sites, and 0, 8, 11 or 19 (8 and 11) amino acids at the z/B site. The insert at the y/A site is required for heparin binding and modulates the binding of agrin to α-dystroglycan, whereas the insert at the z/B site greatly influences agrin signaling at the NMJ[38,42,98]. In the CNS, in contrast, these splice variants seem not to be important for the function of agrin. Agrin contains binding sites for several macromolecular interaction partners, including neural cell-adhesion molecule (NCAM), heparin-binding growth factors, α-dystroglycan, integrins, heparin, α-dystroglycan, LRP4 and the sodium/potassium-ATPase[43,44,96,99,100]. The AChR aggregation activity of agrin has been mapped to the C-terminus. Thereby, a splice insert at the z/B is necessary to exhibit any clustering activity. The C-terminal 95 kDa moiety of agrin is required for full activity in AChR aggregation on muscle cells, but the most C-terminal LG3 domain, comprising of 20 kDa, is sufficient to induce AChR aggregation at low potency[101]. The main source for the agrin isoforms that can induce aggregation is neurons, including motoneurons. Transcripts from all non-neuronal cells, except adult Schwann cells, encode z/B$^-$ agrin.

In the last years, it has become clear that the activities of agrin are of broader significance than

previously anticipated. Agrin is involved in the synapse promotion in the brain, the formation of the immunological synapse, the organization of the cytoskeleton and the amelioration of function in diseased muscle[96].

Agrin is highly expressed in the developing peripheral and central nervous system during the period of synapse formation[27,102,103]. This expression pattern in the brain and the organizing role of agrin at the NMJ indicate that it might orchestrate synaptogenesis in the entire nervous system. Indeed, agrin is important in regulating the formation of cholinergic interneuronal synapses in the superior cervical ganglion (SCG). Agrin-deficient embryos show a mismatch between pre- and postsynaptic structures and have defective synaptic transmission[104]. Importantly, soluble z+ agrin (but not z- agrin) restores the wild type phenotype in cultured agrin-deficient SCG neurons. However, synapse deficits have not been observed in the brain of these agrin-deficient mice, and cortical and hippocampal neurons derived from these animals still form synapses in culture[105,106]. Other studies showed that the clustering of agrin by antibodies[107] and the overexpression of agrin in neural cells induce the formation of filopodia[108], which in turn provide the basis for the formation of new synapses. Dendritic filopodia are thin and usually long membraneous protrusions on dendrites that are involved in synapse formation by establishing preliminary contacts with axonal boutons that potentially mature into persistent synapses within days[109,110]. Acute suppression of agrin expression by antisense oligonucleotides reduces the number of neuron-neuron synapses in cultured hippocampal neurons, which goes in line with decreased filopodia formation[111,112]. Similarly, in adult agrin-deficient mice, when the formation of the NMJ is rescued by transgenic expression of z+ agrin in motoneurons, the number of central synapses is significantly lower than in wild type mice and dendritic arborization of pyramidal neurons is also impaired[113]. Recently, it has been shown that the cleavage of agrin, generating a C-terminal 22 kDa fragment, is necessary for the activity dependent formation of dendritic filopodia in hippocampal neurons[114].

The mediator of the response to agrin may be different at the NMJ and in the CNS. MuSK, which mediates agrin signaling at the NMJ, was longtime thought not to be expressed in the CNS. However, recently, MuSK was found to be expressed in a subpopulation of excitatory synapses and could mediate central response to agrin[113]. Another potential receptor for agrin in the CNS is the sodium/potassium-ATPase. A peptide containing the LG-3 domain of agrin, closely resembling the 22 kDa fragment, was shown to bind to and inhibit the α3-subunit of the sodium/potassium-ATPase. Membrane depolarization and increased action potential frequency in cortical neurons were a direct result of the inhibitory activity of agrin LG3 on the ATPase[100]. Thus, agrin has the potential to regulate activity-dependent processes in neurons, providing a molecular framework for agrin action in the CNS. In addition, the inhibitory effect of agrin on the sodium/potassium-ATPase also modulates the contraction of cardiac myocytes[115].

Recently, a novel role for neuronal agrin in skeletal muscle maturation has been proposed. Agrin appears to control the maturation of the excitation-contraction coupling mechanism in human muscle[116]. The fraction of myotubes showing a functional excitation-contraction coupling was increased in cocultures of muscle cells with spinal cord explants as well as in aneural muscle cells treated with neural agrin. Moreover, the treatment with agrin increased the number of muscle cells in which functional ryanodine receptors and dihydropyridine-sensitive L-type Ca^{2+} channels were detected. These two proteins are essential for functional excitation-contraction coupling. However, these effects have only been observed in human and not in mouse myotubes.

1.3 Neurotrypsin cleaves agrin and destroys its synapse protecting function

The neuronal serine protease neurotrypsin is a member of the serine peptidase family S1A. The murine form of neurotrypsin consists of 761 amino acids with a molecular mass of 84 kDa and a theoretical pI of 8.7, the human form of 875 amino acids with a molecular mass of 97 kDa and a

pI of 8.4. Neurotrypsin is a secreted multidomain peptidase consisting of a signalpeptide (SP), a proline-rich basic (PrB) domain, a kringle (Kri) domain, several scavenger receptor cysteine-rich (SRCR) domains, and a serine protease (Prot) domain at the carboxy terminus (**Fig. 4**). The murine neurotrypsin comprises three, the human form four SRCR domains[117,118]. The peptidase domain has 244 amino acids and shows high similarities to trypsin with 40 % amino acid identity. In front of the peptidase domain is a zymogen activation site with the furin recognition motif RRQKR. Furin is a proprotein converting peptidase, which is widely expressed and processes a broad range of substrates. After activation cleavage, the protease domain of neurotrypsin remains attached to the non-catalytic part via a disulfid bond. Murine neurotrypsin bears three N-glycosylation motives, one in the proline-rich basic and two in the peptidase domain, human neurotrypsin bears only two, one in PrB and one in the peptidase domain[118].

Figure 4 Domain structure of human neurotrypsin. PB, proline-rich basic domain; KR, kringle domain; SRCR1 to SRCR4, scavenger receptor cysteine-rich domains 1 to 4; ZA, zymogen activation region; PROT, serine protease domain; circles, putative N-glycosylation sites.

Neurotrypsin is predominantly expressed in brain, spinal cord, kidney, lung, and testis[118,119]. *In situ* hybridization studies on adult mouse brain revealed a highly restricted expression of neurotrypsin mRNA in the adult nervous system. In the forebrain, neurotrypsin mRNA signals can exclusively be detected in distinct subsets of neurons of the cortex, the hippocampus, the amygdala, and the olfactory bulb. In the neocortex, most prominent neurotrypsin mRNA expression can be found in layers V and VI. Neurotrypsin mRNA is largely absent from the rest of the CNS, except for a strong expression in neurons of the brain stem motor nuclei and the motor columns of the spinal cord. Neurotrypsin mRNA is also present in the peripheral nervous system. A subpopulation of neurons of the dorsal root ganglia expresses neurotrypsin mRNA. Ultrastructurally, neurotrypsin is associated with the presynaptic membrane and the presynaptic active zone of both excitatory and inhibitory synapses of the hippocampus and the cerebral cortex[120,121]. Synaptic recruitment and externalization of neurotrypsin is indcued by neuronal activity, and externalized neurotrypsin lingers at the synapse for several minutes before disappearing because of diffusion or degradation[122]. These observations suggest an activity-dependent function of neurotrypsin at the synapse, such as the activity-dependent remodeling of synaptic circuitries.

During synapse assembly in pre- and postnatal development, neurotrypsin mRNA is upregulated in many brain regions, including hippocampus, neocortex, and midbrain[123]. The peak of neurotrypsin expression, assessed by *in situ* hybridization and western blotting on mouse brain, is between late embryonic and early postnatal phase (embryonic day 12 to postnatal day 10)[121]. A similar pattern for neurotrypsin expression has also been observed in the spinal cord, the peripheral nervous system and in non-neuronal tissues. However, neurotrypsin expression remains detectable during adulthood and is sustained to senescence.

Neurotrypsin was recognized to play an indispensable role for cognitive processes in humans when a four-nucleotide deletion in the coding region of the neurotrypsin gene, which disabled the expression of the proteolytic domain, was identified as the cause of severe mental retardation[120]. The affected children showed normal psychomotor development during the first 18 months but exhibited severe deficits in cognitive development thereafter, indicating that neurotrypsin does not play a critical role for early neural development or for the formation of synapses. Rather, neurotrypsin may be critically involved in the reorganization of synapses and neuronal circuits, which are required to establish and maintain higher cognitive functions during later development and adult stages.

The analysis of transgenic mice overexpressing neurotrypsin in motoneurons led to further insights

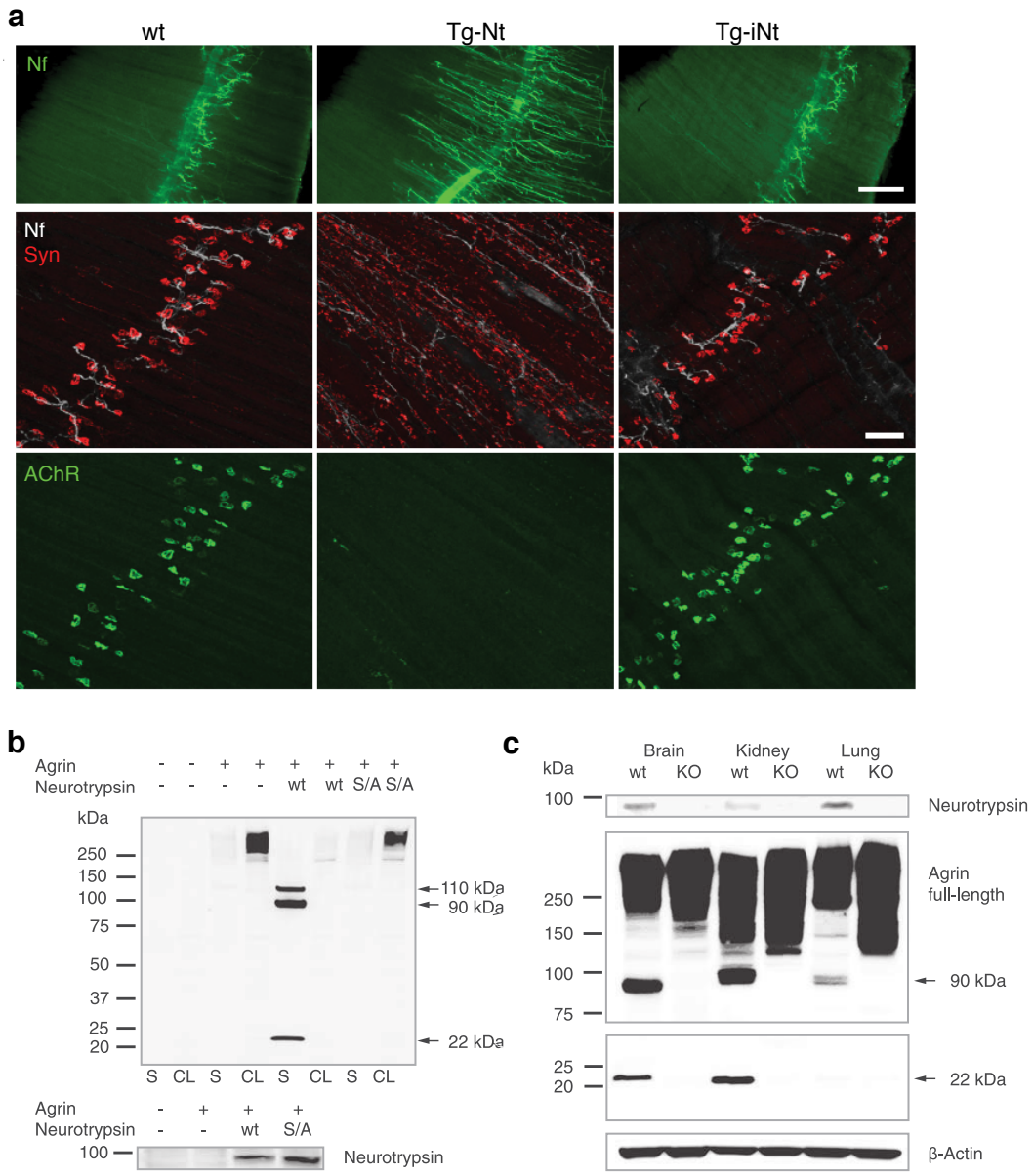


Figure 5 Neurotrypsin cleaves agrin at two positions and destroys agrin's synapse protecting function. (**a**) Overproduction of neurotrypsin in motoneurons causes aberrant innervation of the diaphragm muscle. The neuromuscular innervation of the diaphragm was analyzed by indirect immunofluorescence staining of neurofilament protein (Nf), synaptophysin (Syn), and acetylcholine receptor (AChR), to visualize the motor axons, the presynaptic terminals, and the postsynaptic specializations, respectively. In adult neurotrypsin-overexpressing mice (Tg-Nt), motor axons grew out of the endplate band towards the medial and lateral borders of the hemidiaphragm. Normal NMJs in the endplate band were virtually absent in transgenic mice. Staining for synaptophysin revealed many small, ectopic presynaptic boutons along the trajectories of outgrowing axons. Innervation of muscles from mice overexpressing catalytically inactive neurotrypsin (Tg-iNt) was normal. Scale bars, 500 µm (top), 100 µm (bottom). From Bolliger et al., in preparation. (**b, c**) Neurotrypsin cleaves agrin *in vitro* and *in vivo*. (**b**) Western blot analysis of HeLa cells cotransfected with combinations of membrane-bound agrin (+), wild type neurotrypsin (wt), inactive neurotrypsin (S/A), and empty vector (–). Supernatants (S) and cell lysates (CL) were analyzed with anti-agrin antibodies, directed against the C-terminus of agrin. Transfection of agrin alone resulted in a signal above 250 kDa in cell lysate. Upon cotransfection with wild type neurotrypsin, full-length agrin was cleaved resulting in fragments running at 22, 90, and 110 kDa in supernatant. No cleavage was found after cotransfection with inactive neurotrypsin. (**c**) Western blot analysis of tissue from wild type (wt) and neurotrypsin-deficient (KO) mice. The 90 kDa agrin cleavage product was detected in brain, kidney, and lung of wild type mice but was absent in neurotrypsin-deficient. Similar results were obtained for the 22 kDa agrin fragment except for lung. ß-Actin was used as a loading control. From Reif et al.[119]

into neurotrypsin function. These mice show severe alterations of neuromuscular innervation, very similar to the phenotype found in agrin-deficient mice (**Fig. 5a**). A few days after induction of the transgene, most of the previously established NMJs in the endplate band of the diaphragm muscle had disappeared. Normally positioned NMJs were rare. Motor nerves had grown diffusely over the muscle surface. Along their trajectories they had formed many new, ectopic synapses with small weakly stained AChR clusters. The striking similarity of the abnormal neuromuscular innervation pattern resulting from neurotrypsin overexpression and agrin deficiency, together with the coincident expression of neurotrypsin and agrin in motoneurons suggested agrin as a target of neurotrypsin (Bolliger et al., in preparation).

And indeed, agrin is a proteolytic target of neurotrypsin *in vitro* and *in vivo*. Experiments with the isolated proteins and in cell culture reveled that agrin is cleaved by neurotrypsin at two positions, called α and β cleavage sites (**Fig. 5b**). The first cleavage site (sequence PIER'ASCY) lies in front of the SEA domain, the second (sequence LVEK'SVGD) in front of the third LG domain. α cleavage results in a C-terminal 110 kDa fragment, β cleavage in a C-terminal 22 kDa fragment and the cleavage at both sites results in a 90 and a 22 kDa fragment, respectively (**Fig. 3**). Mutagenesis analysis revealed that neurotrypsin is highly specific due to the unique substrate recognition pocket at the active site[119]. The 90 and the 22 kDa fragments can also be observed *in vivo*, they appear in extracts from brain and spinal cord (**Fig. 5c**). The fragments are increased upon neurotrypsin overexpression in transgenic mice, reflecting an increased agrin cleavage, and are completely absent in neurotrypsin deficient mice. Thus, neurotrypsin is responsible for the cleavage of agrin i*n vivo*. Thereby, only the glycanated variants of agrin are affected. The analysis of agrin cleavage fragments in synaptosomes revealed that the cleavage takes place at or in the vicinity of synapses, resulting in increased concentrations of agrin fragments and decreased full-length agrin at synapses[121].

Neurotrypsin, agrin, and the 90 kDa fragment of agrin, as an indicator of neurotrypsin-dependent agrin cleavage, are highest during fetal and early postnatal stages of neural development and are found at lower levels throughout adult life. Therefore, the temporal pattern suggests a role of neurotrypsin-dependent agrin cleavage in a function occurring with highest activity during neuronal development but persisting at lower activity in the adult brain. In particular, a role of agrin cleavage in developmental and adult synaptogenesis would be well compatible with this expression pattern and with the observation of mental retardation in humans deficient for neurotrypsin.

The C-terminal 22 kDa agrin fragment, which is generated by the proteolytic activity of neurotrypsin, has been shown to be of involved in several processes: it contains the AChR aggregation activity of agrin at the NMJ, inactivates the sodium-potassium ATPase at CNS synpases[100], and induces filopodia formation in hippocampal neurons[114]. The activity-dependent formation of dendritic filopodia was found to be abolished in hippocampal neurons from neurotrypsin-deficient mice. Administration of the neurotrypsin-dependent 22 kDa fragment of agrin rescued the filopodia response. Detailed analysis indicated that presynaptic activity is necessary for the release of neurotrypsin, whereas postsynaptic activity is necessary for the neurotrypsin-dependent agrin-cleavage. Thus, the neurotrypsin-agrin system acts as a coincidence detector of pre-and postsynaptic activation. The region covered by the 90 kDa fragment, on the other hand, interacts with α-dystroglycan, heparin, and integrins[99]. The distinct functions associated with the released fragments indicate major functional differences between full-length and truncated agrin and suggest a role for the proteolytic neurotrypsin-agrin pathway in the regulation of synaptic function required for the establishment and the maintenance of cognitive brain function and the NMJ.

At the NMJ, excessive cleavage of agrin, liberating the diffusible 22 kDa fragment, could inactivate agrin signaling and destroy the synapse protecting function of agrin. The unprotected

NMJs are prone to be dispersed, most likely by an acetylcholine dependent mechanism. Such a process would explain the abnormal innervation pattern seen in neurotrypsin-overexpressing mice, which resembles that in agrin mutants. Importantly, the concomitant transgenic expression of neurotrypsin and a neurotrypsin-resistant agrin variant, due to mutation of the P1 position at both α and β cleavage sites, prevented the expression of the neurotrypsin-dependent phenotype (Bolliger et al., in preparation). This finding confirms that the proteolytic processing of agrin at the α and β cleavage sites is responsible for the disintegration of neuromuscular innervation. Interestingly, the concomitant expression of the cleavage-resistant agrin variant and neurotrypsin delayed NMJ transformation from plaque to pretzel, suggesting that agrin cleavage is important for normal endplate maturation. The cleavage of agrin may relieve agrin-dependent constraint on endplate reorganization during NMJ maturation. Surprisingly, NMJ maturation proceeded normally in neurotrypsin deficient mice, although agrin cleavage has not been found in the brain[114,119,121] and the spinal cord (Bolliger et al.,in preparation) of these mice. Endplate maturation is delayed in the absence of agrin processing at the α and β cleavage sites, but normal in the absence of neurotrypsin. This observation might be explained most directly by the existence of another protease with similar, site-specific activity. This protease should not be expressed in the CNS and exhibit very similar enzymatic characteristics as neurotrypsin, because its proteolytic function should also depend on the presence of the P1 basic residue at each cleavage site of agrin. In summary, the proteolytic processing of agrin at the α and β cleavage sites may regulate the maturation of the NMJ by modifying the extent of the synapse stabilizer agrin in the synaptic extracellular matrix.

1.4 Sarcopenia

1.4.1 Sarcopenia is a severe health problem in the elderly population

Sarcopenia denotes the progressive decline in skeletal muscle mass and function that is associated with aging. It is distinct from wasting, involuntary weight loss due to inadequate energy intake, and from cachexia, cytokine-driven loss of lean body mass that cannot be reversed nutritionally and occurs despite maintenance of weight. Sarcopenia is a consequence of normal aging, and is not necessarily assocated with a disease, although muscle loss can be accelerated by chronic illness. Sarcopenia occurs even in fit, athletic elderly adults, who continue to lose lean mass and muscle mass despite retaining their functional status[124]. Body cell mass is systematically lower in older adults than in middle-aged or young adults, which is largely attributable to a loss of muscle mass[125]. During aging, humans lose about one-third of the skeletal muscle mass. Sarcopenia contributes substantially to impaired mobility and frailty of the elderly and has been associated with an increased risk of morbidity, disability and mortality[126-131]. The prevalence of sarcopenia is almost 25% in humans at the age of 65 years and increases to 30 – 50 % at the age of 80 years[126,132]. However, the rate of sarcopenia varies greatly according to health status, physical activity, and diet. As a consequence, there is a great difference between biological and chronological age, leading to great heterogeneity of elderly subjects and complicating the analysis of sarcopenia.

Due to the increased longevity of industrialized populations, sarcopenia has emerged a major health problem. The estimated direct healthcare costs of sarcopenia in the United States in 2000 were $ 18.5 billion, which represented about 1.5 % of total healthcare expenditures[133].

Sarcopenia not only plays a major role in the loss of muscular strength, but also contributes substantially to decreased metabolic rate[134-138], gradual reduction of bone density[139,140], and decreased aerobic capacity[141,142]. In addition, sarcopenia may also contribute to an increased risk for chronic disease such as diabetes and osteoporosis[143].

Speculations about the pathogenic mechanisms of sarcopenia include a variety of candidate

mechanisms of neurogenic (impaired innervation), muscle intrinsic (inadequate regeneration), or systemic (endocrine failure) origin. Loss of motoneurons and motor unit remodeling[144-146], decreased capacity among motoneurons to innervate regenerating fibers[147], instability of the neuromuscular junction (NMJ)[148], lack of regenerative drive of the muscle, decreased muscle loading[149], physical inactivity[150,151], mitochondrial dysfunction[152,153], oxidative damage[154], excitation-contraction uncoupling[155,156], and decline in satellite cell proliferation and activation[157,158] have been suggested to contribute to sarcopenia as well as nutritional, hormonal, metabolic and immunological factors[159-162]. These factors include age-related declines in growth hormone and insulin-like growth factor-I (IGF-1) production[163], sex steroid levels[164], insulin action, and muscle protein synthesis[165,166]. In addition, fat gain, subclinical inflammation, increased production of catabolic cytokines, and inadequate intake of dietary energy and protein are also potentially influencing the development sarcopenia[127]. Most of these mechanisms may contribute to the pathogenic ensemble resulting in sarcopenia. The relative contribution of each of these mechanisms is, however, poorly understood.

1.4.2 Age-related loss of muscle mass, strength, and quality

The decline of muscle strength in older adults has been demonstrated in a variety of muscles during both voluntary dynamic and isometric contractions[167-180]. However, the size of this age-related decline shows considerable variation among different muscle exercise protocols and sample characteristics. On average, by the seventh and eighth decade of life, maximal voluntary contractile strength is decreased by 20-40% for both men and women in proximal and distal muscles. A longitudinal study of older men (initially 65 years) showed a rate of decline in isokinetic muscle strength of the knee and elbow extensors and flexors of 20 to 30% in 12 years[181]. Vandervoort and McComas[182] showed a pronounced decline of up to 50% for maximum voluntary strength of the ankle dorsiflexor and plantarflexor muscles in 111 men and women, starting in the 6th decade (**Fig. 6**). Stimulation of motor nerves during maximum voluntary effort produced no additional force in the majority of the elderly men and women, indicating that these subjects remained able to utilize their descending motor pathways for optimal muscle activation. Therefore, a decrease in excitable muscle mass appeared to be entirely responsible for the lower strength in the elderly, a hypothesis that will be discussed below. Recently, McNeil et al.[183] demonstrated that not only static muscle strength but also velocity-dependent muscle power (work over time) is substantially reduced with age. Age-associated decreases in velocity induced reductions in power that were two to three times the magnitude of losses in strength. In a large study including 1030 subjects in the age range of 20-102 years, an age-related decline in knee-extension torque, handgrip strength, muscle power, and muscle thickness was found[184]. Reductions of strength were between 50 and 60% in the oldest (older than 85 years) compared to the youngest (20-29 years old) age group for both men and women. Muscle power, however, dropped by about 75%, whereas calf muscle area was only 15 and 31% lower in the aged women and men, respectively. Low muscle strength and power were strongly associated with poor mobility, whereas a small calf muscle cross-sectional area (CSA) was a

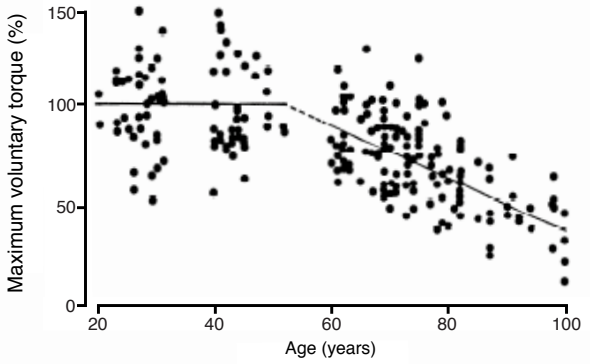


Figure 6 Age-associated change of maximum voluntary dorsiflexor and plantarflexor torques, expressed as percentage of mean values for youngest group (20-30 years). From Vandervoort and McComas[182].

weak predictor of mobility limitations.

The contractile properties of muscles from aged rodents for the most part parallel the findings of human muscle. Here, the contractile properties can be analyzed *ex situ*, in single muscle preparations. Arabadjis et al.[185] showed that the peak twitch tension, rates of force development and relaxation are significantly lower in old than in young rat plantaris muscle. Brooks and Faulkner[186] demonstrated that the maximum titanic force of m. soleus and m. extensor digitorum longus (EDL) of 26 months old mice were 78 and 73%, respectively, of the values for 9 months old mice.

The majority of muscle strength loss in age can be accounted for by decreased muscle mass. Muscle volume and muscle cross-sectional area of humans have been measured by various indirect and direct methods. A number of ultrasound, computed tomography, magnetic resonance imaging (MRI), and direct cross-sectional studies have quantified the decline in skeletal muscle mass with age[170,171,177,180,182,187-189]. Muscle mass has been demonstrated to decrease by up to 40% between the ages of 20 and 70 years. Young et al.[170,171] reported for example 25-35% reductions in the CSAs of the quadriceps muscles of older men and women compared with young controls using ultrasonographic imaging. Isometric strength of the quadriceps muscle was also reduced and a clear correlation of muscle thickness and strength has been found in the old subjects. Computed tomography scanning has shown similar results for the quadriceps and the biceps brachii muscles[180,190]. More recently, two studies used larger sample sizes and measured whole skeletal muscle mass instead of only some individual muscles. Gallagher et al.[191] measured total appendicular skeletal muscle mass using dual-energy X-ray absorptiometry in 148 women and 136 men between 20 and 90 years of age and found an age-related decrease in total appendicular skeletal muscle mass of 10-15%. Janssen et al.[192] determined skeletal muscle mass in 468 men and women between 18 and 88 years of age using whole body magnetic resonance and found an age-related decrease in muscle mass of up to 20% after the end of the fifth decade. Frontera et al.[181] accomplished a 12-yr longitudinal study on men initially 64 years old and measured the CSA of all thigh muscles, the quadriceps femoris muscle and flexor muscles by computerized tomography. A reduction of about 15 % in muscle CSA was found over the 12 years. Furthermore, this and other studies have shown a correlation between reduction in muscle mass and strength[167,170,171,177,179,193,194].

Age-associated reduction in muscle mass has not only been observed in humans but also in rodents, although there has been considerable variability in this response between different studies. In an early report, Tauchi et al.[195] showed a weight reduction of about 50% of the tibialis anterior muscle of 24 months old compared to 12 months old rats. Similar results were obtained from Tucek and Gutmann[196]. Muscle masses of EDL, soleus and diaphragm in 28 months old rats were reduced 47, 56 and 19%, respectively compared to 9 months old animals. Soleus and EDL muscles of the rat have been extensively analyzed for age-related loss of mass in a number of studies. A decline in muscle mass of 10-30% in aged (24-30 months) compared to adult (8-12 months) animals has been observed[197-201]. However, the extent of the age-associated loss of muscle mass and the age of onset varies considerably between the different studies. This effect is due in part to the very wide range of ages used to represent old animals, and perhaps the confounding influence of pathology in the inbred strains typically studied in the past. More recently, survival curves for specific rodent strains have been used to more accurately describe the relative ages of the animals studied. In this respect, the Fischer 344xBrown Norway F1-hybrid rat has been shown to demonstrate progressive skeletal muscle atrophy with aging on a consistent basis[202-205]. The observed decline in muscle mass is very similar to the effects in humans in the analogical age-range.

Interestingly, the decrease in muscle strength in older subjects appears to be greater than the decrease in muscle CSA, suggesting that the quality of skeletal muscle or efficiency of muscle strength per

muscle mass is reduced with age[166,180,183,186,194,206-208]. Kallman et al.[179] measured grip strength and muscle mass in 847 healthy humans, aged 20-100 years. Both cross-sectional and longitudinal results indicated that grip strength increases into the thirties and declines at an accelerating rate after 40. Grip strength was strongly correlated with muscle mass, but multiple regression analysis showed that grip strength is more strongly correlated with age than with muscle mass. Further analysis demonstrated that younger subjects were stronger and older subjects were weaker than predicted based on their muscular size. Thus, decline in muscle mass does only partially explain the loss of force seen with aging. This finding is supported by the study of Vandervoort and McComas[182], reporting a significant decline of plantarflexor muscle strength relative to CSA in old (82-100 yr) compared to young (23-31 yr) men and women. The authors suggested that muscle denervation and increased amounts of connective tissue in the muscles of elderly subjects could be responsible for a specific decrease in excitable muscle mass. However, age-associated decline in specific force (force per muscle CSA) was also observed after correction of the CSA for increased amounts of non-contractile tissue in older subjects[209]. The change in muscle CSA accounted for only about half of the change seen in force production, suggesting a loss of contractile muscle quality. In another study, maximum voluntary force and CSA of the adductor pollicis muscle were compared in 53 young (19-55 years) and 39 elderly (72-90 years) subjects[210]. Despite both young and elderly subjects were able to fully activate their muscles, the specific force (voluntary force per muscle CSA) was 26% lower in the old group. Thus, the muscle weakness of old age did not appear to be due to reduced activation of the muscles. Findings from rodent studies resemble those from humans. Brooks and Faulkner[186] found a specific force deficit of 22% for the EDL muscle of aged animals, suggesting a decline in muscle quality. However, the specific force of the soleus muscle did not change significantly in the observed age range, although the same tendency was noticed. An age-associated decrease in peak absolute force and specific tension (peak absolute force per CSA) was even demonstrated for single muscle fibers of aged (24-37 months) compared to adult (12 months) rats[204]. This finding was confirmed in more recent studies, reporting a reduction of specific force generated by single muscle fibers of the EDL, the soleus and the flexor digitorum brevis muscles of 26%, 19% and 37%, respectively in old (20-24 months) compared to young adult (2-6 months) mice[211,212]. However, these findings are challenged by a number of studies. Brooks and Faulkner[213] did not find reduced specific force in single muscle fibers from EDL muscles of aged (27 months old) compared to adult (12 months old) mice. Thus, the age-associated decrease in muscle strength was not the result of an impairment of the intrinsic force-generating capacity of single muscle fibers. Similarly, age-related force deficits were not found in living fiber bundles and skinned fibers from EDL and soleus muscle of rats[214]. Changes in the muscle itself, either in the contractile proteins or in excitation-contraction coupling, appeared not to be responsible for the weakness in old age, which instead was likely due to changes in structures or processes external to the muscle, such as alterations in neuromuscular transmission. Together, a decline in absolute and specific force with aging is generally accepted, but the contribution of reduced force generation of single muscle fibers is still conversely discussed. The age-associated loss of specific force may not be simply explained by reduced force generation of single muscle fibers, although this may be a contributing factor.

There have been extensive discussions about the mechanisms underlying specific force losses with aging. Contraction-induced injury[215,216], appearance of denervated muscle fibers[208] (see below), infiltration of fat and connective tissue (see below), decreased muscle protein synthesis rate[166] (see below), age-related posttranslational modifications of contractile proteins[217,218], and excitation-contraction uncoupling (ECU)[155,219] have been suggested to contribute to age-associated loss of muscle quality.

Recent evidence supports ECU as a key factor explaining the discrepancy between age-related loss

of muscle mass and muscle strength. The uncoupling between excitation of the muscle and increase in myoplasmic Ca^{2+} concentration is thought to be responsible for ECU. It has been demonstrated that the peak intracellular Ca^{2+} evoked by sarcolemmal depolarization under voltage-clamp conditions in muscle fibers from old mice is significantly smaller than that recorded in fibers from middle-aged or young mice[219]. This substantial reduction in intracellular Ca^{2+} accumulation upon fiber activation leads to ECU, because the level of free cytosolic Ca^{2+} regulates muscle tension. Gonzalez et al.[212] measured intracellular Ca^{2+} concentration and contractility simultaneously, and found a direct relationship between the decreases of both. Impaired Ca^{2+} mobilization following muscle excitation results in decreased muscle contraction. Two key molecules involved in intracellular Ca^{2+} handling and muscle contractility are the dihydropyridine (DHPR) and ryanodine (RyR1) receptors. The DHPR is a dihydropyridine-sensitive voltage-gated L-type Ca^{2+} channel that induces Ca^{2+} release from the sarcoplasmatic reticulum (ST) through the activation of ryanodine-sensitive Ca^{2+} release channel (RyR1)[220,221]. The transduction of the trans-sarcolemmal potential into elevations in intracellular Ca^{2+} is a key event that induces muscle contractions. Absolute reductions in the number and/or function of the DHPR and/or RyR1 are potentially causing the age-related impairment in intracellular Ca^{2+} mobilization in skeletal muscle. The number of DHPR and RyR1 expressed in mouse EDL and soleus muscles indeed decreases with age[222,223]. IGF-1 could play an important role in this process by modulating the expression of DHPR in skeletal muscle[224]. Transgenic overexpression of IGF-1 in skeletal muscles of mice resulted in increases in DHPR and RyR1 mRNA in young and old mice and prevented age-dependent decrease in intracellular Ca^{2+} in skeletal muscle fibers. Thus, specific force of single muscle fibers and the whole muscle could be maintained at older age at a level similar to young muscles[212]. Alternatively, reduced homoestatic capacity for intracellular Ca^{2+} movement may underlie ECU[225]. The dynamic nature of Ca^{2+} sparks seems to disappear in aged skeletal muscle, most likely due to the uncoupling of a segregated Ca^{2+} reserve from the normal EC process. A similar phenotype can be observed in mitsugumin-29 (MG29) mutant adult muscles. MG29 is involved in maintenance of muscle membrane ultrastructure and Ca^{2+} signaling and its expression is decreased in aged muscles, suggesting that MG29 is important in maintaining skeletal muscle Ca^{2+} homoestasis during aging. In summary, ECU may explain the decrease in skeletal muscle force, which can't be attributed to muscle atrophy. ECU is most likely caused by age-dependent reduction in charge movement and peak cytosolic Ca^{2+}, which are associated with decreased DHPR and RyR1 gene expression, and can be prevented by skeletal muscle IGF-1 overexpression.

The loss of muscle quantity and quality is associated with a loss of function in task performances of daily living, such as basal movement in the upper extremities in hygiene and dressing activities, ability to rise from a stool, and stair climbing[226]. Bassey et al.[227] demonstrated that leg extensor power correlated with multiple functional parameters, including stair climbing speed and walking speed. In agreement with this finding, there is a close correlation between loss of quadriceps muscle strength, loss of skeletal muscle mass, and slowing gait speed in frail institutionalized men and women over 86 years of age[193]. Similarly, Lauretani et al.[184] found sarcopenia to be associated with low walking speed and the inability to walk at least 1 km without difficulty in a large study including 1030 persons. These functional impairments mean a loss of independence for many individuals suffering from sarcopenia.

1.4.3 Age-related morphological changes in skeletal muscles

Each skeletal muscle contains many contractile cells (myocytes) that are commonly referred to as muscle fibers. Skeletal muscle fibers can be categorized into several types on the basis of which isoform of the myosin heavy chain is expressed. Type I fibers, slow-twitch fibers, develop force

relatively slowly in response to activation and are characterized by an ability to sustain tension development over prolonged period of times. They are characterized by high oxidative capacity that is mediated by high mitochondrial density and by low glyocolytic capacity. Type II fibers, fast twitch fibers, are capable of faster contraction in response to activation. In humans, they can be further categorized into two types. Type IIx fibers contract very fast and produce high power but show low resistance to fatigue and low mitochondrial density. Type IIa fibers exhibit intermediate characteristics between type I and type IIx. Many other animals express a third subclass of type II fibers: type IIb fibers, which usually exhibit the fastest development of force in response to activation and exhibit the least resistance to fatigue during repetitive activation. Different fiber types express distinct myosin heavy chain isoforms. The ATPase of these myosin isoforms exhibit different stability towards acid- or alkali treatment, a characteristic that has been used for histological discrimination of the fiber types[228,229]. Cross-reinnervation experiments demonstrated that the fiber type is determined by the innervating motoneuron and that all fibers are principally capable of expressing all myosin isoforms[230].

1.4.3.1 Loss of muscle fibers is a major pathologic characterstic of sarcopenia

Over the past three decades, it has become generally accepted that loss of muscle fibers is probably a major pathologic feature of sarcopenia[231]. The marked decrease in muscle fibers with advanced age has extensively been documented. Lexell et al.[232] showed an age-related loss of muscle fibers in a comprehensive study of the whole m. vastus lateralis in 43 male cadavers at age between 15 and 83 years. The number of fibers was reduced by about 50% over this age range. The decline in fiber number was already evident at the age of 30 years and increased progressively. After the age of 50 years, an average annual fiber loss of over 1% was registered (**Fig. 7**). All studies in humans indicate that muscle mass is well maintained throughout the fifth decade, but considerably and progressively diminishes thereafter.

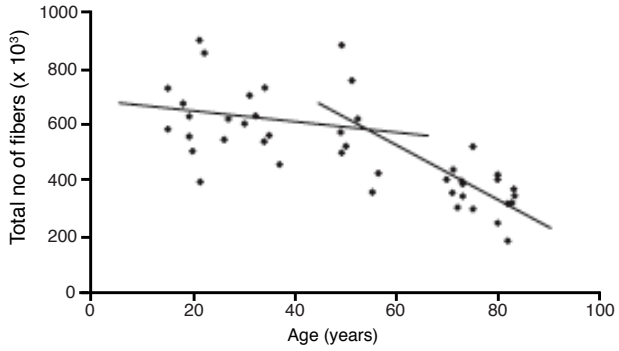


Figure 7 Relationship between the total number of fibers in the vastus lateralis muscle and the age of men between 18 and 82 years of age. The number of fibers does not change between 18 and 50 years of age, but decreases thereafter. By age of 80 years, the mean number of fibers drops to 50% of the number for younger men. Modified by Faulkner et al.[236] from Lexell et al.[232]

Most of the knowledge about fiber numbers comes, however, from animal studies. In rats, two muscles of the lower extremities, the slow-twitch soleus and the fast-twitch EDL muscle, have been extensively analyzed in regard to age-associated change in fiber numbers. Decreased number of fibers in the senescent soleus muscle has been constantly found in a large number of reports[146,196,197,199,201,233-235], although the extent of fiber loss varied greatly between the studies. Overall, a reduction of about 10% in the number of fibers at the age of 24 months and more than 30% at the age of 30 months has been reported. This appears to be less than in humans, which has been attributed to the reinnervation process that could be more effective in the smaller muscle of rats[236]. A minority of studies did not reveal a significant age-related decrease in fiber number of the soleus muscle, although such a tendency was noticed[200,237]. Age-related decrease in the number of muscle fibers has also been reported to occur in the rat EDL muscle[196,199] and has again be challenged by studies that have found a constant number of fibers during aging[146,200,235,237]. Finally, Wanagat et al.[152] reported an age-related loss of 30 % of the fibers of the rat rectus femoris muscle and Tauchi et al.[195] observed such a loss in tibialis anterior and psoas

major muscles. Conflicting results and discrepancies are probably ascribable to variations between different strains and ages of the animals, as well as to methodological differences. Together, the vast majority of the studies indicate an age-related loss of fibers in different rat muscles. However, the extent of this fiber loss and muscle specific differences are not fully understood.
There are still ongoing discussions about the relative contribution of muscle fiber loss and atrophy to age-related decline in muscle mass. Both the number and thickness of individual muscle fibers can be reduced with age. However, individual muscle fibers have also been shown to undergo compensatory hypertrophy with aging, suggesting that a part of the muscle fibers have the abilities to grow in age. Hepple et al.[238] reported that the mean fiber CSA of the soleus muscle was actually increased in aged compared to adult rats. Hypertrophy of muscle fibers can also be induced with physical exercises in young and aged people (see below). Even when age-related fiber atrophy is diminished after physical exercises, age-associated loss of muscular strength can be observed. Thus, the strength decline in age may not primarily be due to muscle fiber atrophy but due to muscle fiber loss. Fiber atrophy could be introduced by non-functional, degenerating fibers or could be secondary to age-related decrease in activity, potentially caused by developing sarcopenia. Nevertheless, the impact of fiber atrophy may be quite severe, especially without exercising. Daw et al.[199] analyzed soleus and EDL muscles of 27 months old rats and found that the fiber numbers declined with age only by 5.6 and 4.2%, respectively. Muscle masses, however, were decreased 17 and 30%, respectively. The authors concluded that the loss of muscle fibers could account at most for about 25% of the observed skeletal muscle atrophy.

1.4.3.2 Fiber type specific alterations in aged skeletal muscles

It is generally agreed that the averaged size of type II fibers is considerable reduced with increasing age in humans, while the size of type I fibers remains more or less unaffected[232,239-245]. Coggan et al.[240] reported for example a reduction of 13-31% in type IIa and type IIb fiber areas in the gastrocnemius muscle of older men and women compared to young adults, whereas the area of type I fibers remained constant.
The proportion of type I and II fibers was extensively analyzed for age-related change. Here, the situation is less clear. Based on early studies of muscle biopsies from humans, a preferential loss of type II fiber with aging was postulated[242,243,246,247]. These findings have been challenged in other biopsy studies that have not found a type I preference in senescent muscles[239,248,249]. However, a muscle biopsy sample consists of, at most, several hundred out of thousands muscle fibers that form a muscle, and the sample-to-sample variability of fiber type with this method of analysis is well established and may explain the conflicting results. Therefore, a single biopsy is a poor estimator of the fiber type proportion of a whole muscle[250]. Finally, an exhaustive analysis of cross-sections, using whole muscle taken from human cadavers, did not reveal a preferential loss of type II fibers, although such a tendency was observed[232]. However, there is considerable variability between individuals of similar ages; for example, some individuals have large type II fibers, while others have a high proportion of type II fibers. Together, both type I and type II fibers may be equally lost in humans. However, the relative type I area is increased, due to selective type II fiber atrophy.
In animal studies, predominant reduction of type II fiber thickness has also been observed. Alnaqeeb and Goldspink[237] found a significant decline in the CSA of type II fibers in soleus between rats aged 10 months and 24 months, but no reduction in size of type I fibers. In the rat plantaris muscle the CSA of type II fibers declined 37% and the type I fibers only by 21% between ages of 9-10 months and 28-30 months[251]. In contrast to humans, an increase in the relative number of type I fibers in aged rodents is generally accepted. Several studies reported an increase in type I fiber proportion in the soleus muscle of aged rats[146,201,234,235,252-255]. Thereby, studies using myofibrillar ATPase staining or type specific MHC antibodies were in close agreements. Larsson and Ansved[256] showed that the

mean percentage of type I fibers in the soleus muscle increased from 71 in 2-month old rats to 98 in those 12 months old or older, based on myofibrillar ATPase staining characteristics. Consequently, a clear age-associated reduction in the number and proportion of type II fibers was noticed. Age-associated type I fiber preference was also observed in the diaphragm, tibialis anterior, rectus femoris muscles, but not in the EDL muscle, which contains only a very low number of type I fibers[200,234]. Recently, age-related increase in the proportion of type I fibers has also been found in the medial gastrocnemius muscle of rats[257]. Together, the majority of studies about a variety of rodent muscles reports the accumulation of type I fibers in senescence. However, the extent of the fiber type rearrangement is poorly understood. Conflicting results between different studies do not allow a clear readout. Eddinger et al.[200] summarized the fiber type composition of different rat muscles, which had been presented in a large number of distinct studies. The percentage of type I fibers in the soleus muscle of adult male rats, for example, varies between 66 and 90%, depending on the strain and the experimental method. With this differences in mind, it is not surprising that the results concerning age-related changes in fiber type composition vary considerably.

Several ideas have been proposed to explain the apparent reduction in the proportion and thickness of type II muscle fibers with aging. Central to these ideas is the hypothesis that some characteristics of type II fibers render them more susceptible to aging-induced processes, resulting in their preferential loss or atrophy. For example, mitochondrial dysfunction, which arises from the accumulation, over time, of mitochondrial DNA damage as a result of oxidative stress, leads to fiber atrophy in rats[152]. The lower mitochondrial volume of type II fibers could render such a cell more susceptible to death via mitochondrial dysfunction mechanisms[153]. Another aspect is the transformation of type II to type I fibers, which could contribute to the increase in the proportion of type I fibers. This process includes the denervation and subsequent reinnervation and reprogramming of muscle fibers and results in fiber type grouping, processes that can be observed in aged muscles (see below). Reinnervation by type I motoneurons may occur more often than reinnervation by type II motoneurons, resulting in an increase in type I fibers. Different stabilities of the motoneurons as well as the corresponding NMJs towards age-associated degeneration processes could be the reason. An alternative hypothesis is independent of innervation: Vaughan et al.[258] speculated about an influence of androgens on the percentage of type I fibers, since this percentage was higher in female than in male mice, but was similar between castrate males and females. Interestingly, even castration at an age of 4 month, an age by which the “male” pattern of fiber types should have been fully established, resulted in a significant increase in type I fibers. It therefore appeared that continuously high androgen levels are required to maintain the low proportion of alkali-labile type I fibers. With age, levels of androgens are decreased in both males and females[259-261], potentially inducing an increase in type I fibers.

1.4.3.3 Age-associated fiber type grouping implies neuropathological processes

The light microscopical observation of groups of histochemically similar muscle fibers, referred to as fiber-type grouping, is commonly considered to be evidence of a denervation and reinnervation process affecting the spinal motor neurons or the peripheral nerves (**Fig. 8a**). In healthy muscles, the two fiber types are randomly arranged, and the term mosaic is commonly used. The occurrence of large groups of fibers with the same histochemical properties provides evidence of a neuropathological process (**Fig. 8b**).

Muscle fibers belonging to one motor unit have similar structural and functional properties and intermingle with fibers of other motor units. Denervation of muscle fibers, resulting from motor neuron death, from irreparable damage to peripheral nerve axons, or eventually from deterioration of the NMJ, is followed by the growth of fine sprouts from nearby axons. When these sprouts establish new contacts with the denervated fibers (reinnervation), the size of the remaining motor

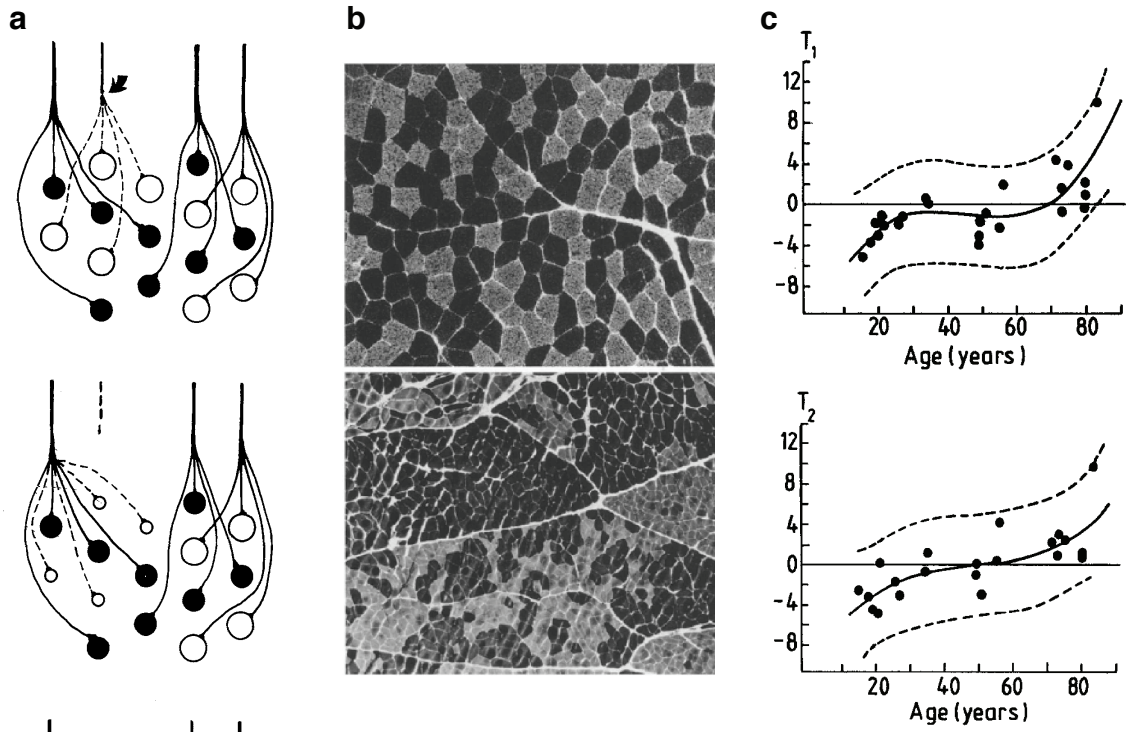


Figure 8 (**a**) Schematic illustration of the process of fiber type grouping. Muscle fiber denervation (arrow) and reinnervation, with the subsequent fiber-type transformation leads to the change in the arrangement of fiber types known as fiber-type grouping. From Lexell et al.[640] (**b**) Small parts of whole cross-sectioned autopsied vastus lateralis muscle from a healthy young man showing the two fiber types, type 1 (lightly stained) and type 2 (heavily stained), in a mosaic pattern (top), and a young man with spinal muscular atrophy, showing excessive grouping of both fiber types (bottom). Stainings for myofibrillar ATPase at pH 9.4. From Lexell and Downham[267]. (**c**) The measures T1 and T2 of the arrangement of type 1 and type 2 fibers for the vastus lateralis muscle from 24 men plotted against age with the cubic regression line (continuous line) and 95 % prediction interval (dashed lines). Fiber type grouping, represented by positive T values, can be observed beyond 60 years of age. From Lexell and Downham[267].

units can increase more than tenfold. When reinnervation occurs, muscle fibers completely adapt to the functional demands mediated through the new nerve. For example, if denervated type II fibers are reinnervated by type I neurons, they are transformed to type I fibers. A progressive denervation and reinnervation process may in this way ultimately change the arrangement of fiber types and so cause the clustering referred to as fiber type grouping. Thus, fiber type grouping is used with other histological features (angulated fibers, grouped atrophy, etc.) as evidence of an underlying continuous neurogenic disorder.

Early studies suggested that aging is associated with fiber type grouping[198,241,245,247,262-264]. However a statistical method to analyze fiber type grouping was not available. Lexell and colleagues adopted the measure of counting the number of enclosed fibers[262] and developed a statistical method to assess fiber type grouping using a model based upon hexagonal-shaped fibers[265]. They found increased type grouping with age in three groups of men at the age of 24, 52 and 77 years[266]. For the old group, the proportion of fascicles with a significant number of enclosed fibers (21%) was greater than the proportion for the middle (6%) and the young group (6%). Increasing age affects the fiber type arrangement, leading to an increased occurrence of enclosed fibers. This finding was confirmed, using an improved statistical and analytical method[267]. This method allowed the calculation of the expected number of enclosed fiber, when a random distribution was assumed. Thereby the influence of the fiber type proportions and positions were considered. Comparison of

the expected and the counted number of enclosed fibers gave the possibility to make a statement about the randomness of the fiber type distribution. Using this method, it has been shown that in the vastus lateralis muscle of persons up to 25 years of age, the fiber types are segregated. From the ages of 30 to 60 years, fiber types are randomly arranged and above the age of 60 years, fibers of both types are grouped (**Fig. 8c**).
Edström and Larsson[146] used another method to determine muscle fiber rearrangements in the old motor units of the rat. Soleus muscle fibers belonging to a motor unit were identified using the glycogen depletion. The principle of this method is to repeatedly stimulate a single motor unit until the fibers are depleted of glycogen. These fibers are then mapped as unstained fibers in PAS (periodic acid-Schiff reagent)-stained sections. To assess the randomness of the fiber arrangement the muscle was divided into eight territories, which should contain equal numbers of depleted fibers. The mean difference between measured and predicted numbers of fibers in the eight sectors of the motor unit territory was calculated in young (3-6 months) and old (20-24 months) rats. In the fast-twitch units, these differences were not affected by age, whereas in the slow-twitch unit the differences were increased in the old compared to the young animals, indicating a rearrangement of the muscle fibers in the old slow-twitch motor units. These findings confirm previous morphological studies of muscles from old individuals in which indirect or direct signs of a neurogenic process have been presented. Type grouping does not occur in aged long-term denervated muscle, confirming that the grouping in old skeletal muscle is a sign indicative of denervation and reinnervation processes under control of the nervous system[268].
The collected evidence strongly indicates that the muscle fiber population undergoes continuous denervation and reinnervation with increasing age. The etiology of denervation is considered to involve motor neuron death, irreparable damage to peripheral nerve axons, or, eventually, deterioration of the NMJ. This age-related progressive neurogenic process must be a major contributor to the gradual loss of fibers with increasing age.

1.4.3.4 Increase in heterogeneity of fiber thickness indicates coincidental hypertrophy and atrophy in sarcopenic muscles

As early as 1968 an abnormal degree of variation in fiber CSA has been observed in muscle biopsies of elderly subjects[263]. Later, an age-associated increase in the heterogeneity of fiber thickness was confirmed in numerous studies in humans, rats, and mice[175,198,237,244,269,270]. The proportion of both, severely atrophied and hypertrophied muscle fibers is increased in senescent muscles and contributes to the picture of increased heterogeneity of fiber thickness. Recently, Hepple et al.[238] showed differences in the age-related alterations of fiber sizes between individual skeletal muscles of aged rats. Atrophied muscles were found both in the gastrocnemius, a locomotor muscle, and in the soleus, a postural muscle. Hypertrophied fibers, however, were only observed in the slow region of the gastrocnemius and in the soleus muscle. Frequency histograms underscored the increase of heterogeneity with aging, as they demonstrated the presence of both atrophied and hypertrophied fibers in soleus muscle, and atrophied fibers in gastrocnemius muscle in aged compared to young animals. Interestingly, the atrophied fibers of the soleus muscle exhibited a reduction of subsarcolemmal mitochondrial staining, an observation suggesting the denervation of these fibers. Denervation may result in atrophy of the corresponding fibers due to the loss of neuronal input. Functionally innervated fibers, on the other hand, may undergo hypertrophy to compensate for the loss of contractile area. This compensatory effect is likely to correlate to the load on the muscle. The use of a locomotor muscle, such as the gastrocnemius, might be reduced in age, as reduced physical activity is a common and often observed consequence of aging. Decreased physical activity does not reduce loading on a postural muscle, such as the soleus, to the same extent. Therefore, compensatory hypertrophy may be more pronounced in soleus than in

gastrocnemius muscle.

1.4.3.5 Accumulation of angular and central nucleated muscle fibers and infiltration of non-contractile tissue in aged muscles

Further histological features of skeletal muscles in old age have been described in a number of early papers[262-264]. Grouped atrophy, fiber splitting, centrally located nuclei, areas of focal necrosis with infiltration of macrophages, and replacement of muscle fibers by connective tissue or by fat has been observed in muscle biopsies of aged subjects. Tomonaga[245] described several neuropathic and myopathic changes in senile human skeletal muscle. Neuropathic changes included small, angulated fibers, type grouping, grouped atrophy, target fibers, and nuclear clumps, whereas myopathic changes included central nuclei, necrosis, phagocytosis, and interstitial fibrosis. The neuropathic changes were found to predominate, and thus, denervation processes were suggested. Increased occurrence of angulated atrophic fibers was found in both aged EDL and soleus muscle of the rat, together with central nucleation of muscle fibers and fiber splitting[197,198]. Angular fibers are considered to be evidence for denervation[158,197], suggesting the denervation of muscle fibers as pathogenic mechanism underlying age-related fiber loss. Myopathic changes, such as centralized nuclei, could be reactions of the muscle to the denervation induced muscle degeneration. In human anterior tibial muscle, the frequency of central nucleated fibers increased form 1.1% to 10.3% in elderly compared to young subjects[242]. In rat soleus muscle, the number of centrally located nuclei per 100 fibers increased from about 2 in 6-month-old animals to more than 12 in 32-months-old animals[271]. Centrally nucleated fibers are commonly recognized as regenerated fibers, since newly formed muscle fibers have central nuclei[272]. Thus, a regeneration reaction of the muscles to the aging-induced loss of fibers can be assumed. The infiltration of fat and connective tissue, replacing muscle tissues might be a result of fiber atrophy and the ongoing degeneration and regeneration process[167,190,197,273]. Kent-Braun et al.[274] quantified contractile and noncontractile contents of the leg anterior compartment. Young adults had larger contractile areas and smaller absolute and relative noncontractile areas than older subjects. The content of noncontractile tissue increased from 6% in both young women and men to 13.9 and 15.9% in older women and men, respectively. A 2- to 3-fold increase in intramuscular noncontractile content in older adults was also found in the quadriceps[190,209,275], in the hamstrings[190] and in the elbow extensors and flexors[189]. Age-related increase in endomysial connective tissue of the soleus and EDL muscles has also been observed in rats[198,276]. Perimysial connective tissue, in contrast, remained constant throughout life.

In summary, different neuropathic and myopathic changes can be observed in aged skeletal muscles. Predomination of neuropathic changes suggest that degenerative changes of muscle fiber innervation could induce the pathogenic process resulting in sarcopenia. Myopathic changes might represent muscle intrinsic reactions to the neurogenically induced atrophy or loss of myofibers.

1.4.4 Fiber regeneration and de novo generation in adult and aging skeletal muscle

The number of muscle fibers an adult will have is largely determined during prenatal development by the process of myogenesis. Mesodermal germ cells differentiate into myoblasts, which fuse and form myotubes. Myotubes then differentiate into mature muscle fibers. This process is largely complete before birth. Muscle growth between early childhood and adulthood occurs exclusively by increase in thickness of the individual muscle fibers. Skeletal muscle fibers are postmitotic, thus loss of fibers, for example due to aging, should result in a permanent reduction in the number of fibers. However, it is now well established that skeletal muscle contains mitotically competent cells that contribute not only to muscle fiber hypertrophy in response to physiological overload but also to the regeneration of muscle fibers after other types of damage. These mitotically competent

cells are referred to as satellite cells[277]. They are mononucleated and located between sarcolemma and basement membrane of terminally-differentiated muscle fibers. Satellite cells determine the regenerative capacity of skeletal muscle after injury, surgery or in neuromuscular diseases involving muscle degeneration such as muscular dystrophies. Among the parameters involved in muscle regeneration, the number of satellite cells, their proliferative capacity, and the speed at which the general response to injury is induced all influences the recovery.

Whereas fiber number is known to decrease with aging, the extent to which fibers are regenerated after age-related loss is unclear. Obviously, if regeneration occurs, it is not enough to keep up with the loss. In adult mammels, induced myocyte necrosis, for example by injection of bupivacaine, is followed by regeneration. Bupivacaine injection causes dissolution of the sarcolemma that leads to rapid fiber necrosis but does not affect basal laminae, satellite cells, intramuscular nerves and blood vessels. Interestingly, regeneration after injury restores a very similar number of muscle fibers as were present before necrosis, suggesting that myocyte number is tightly regulated[278]. Thus, with aging, the regulation of myocyte number itself or the capacity for regeneration becomes compromised. The later could for example be caused by reduced number or reduced proliferative potential of satellite cells as well as impaired activation and incorporation of satellite cells into myotubes, or unsuccessful innervation of newly generated myotubes.

Satellite cells proportion were estimated at 15 % of all myonuclei at birth, 6-10% at 2 years of age, and only 4% in the adult[157,279,280]. For aged subjects, this value varied between 0.6 and 3.4% in independent studies[157,280,281]. Decreased number of satellite cells was accompanied by increased number of myonuclei per muscle fiber in elderly subjects[282]. This finding suggests increased incorporation of satellite cells into myofibers without adequate restorage of the reserve pool. However, telomere lengths, an indicator of the regenerative history, were not changed, suggesting that there is only a limited incorporation of new myonuclei originating from satellite cells during normal aging[157]. The decrease in numbers of satellite cells could be due to a continued low level turnover of the satellite cells, which occurs during normal growth and repair, and which exhaust the proliferative capacity of some of these cells. Consequently, they can no longer be activated and the size of the reserve pool would decrease. An exhausted pool of satellite cells was also been demonstrated in long–term denervated muscles. The percentage of satellite cells increased at 2 month of denervation, but substantially decreased thereafter[268,283].

Therefore, in concert with reduction in number of satellite cells with age, decreased proliferative potential has been expected. Indeed, age-related decline in satellite cell proliferative potential has been reported[284,285]. Under identical *in vitro* conditions, the ability of satellite cells to proliferate and form colonies was inversely proportional to the age of the donor animals. Thus, a possible limited proliferative capacity of satellite cells of senile animals must be considered as a contributory factor to atrophic changes in the skeletal muscle. However, the main difference in proliferative potential was seen between cells from young (6-30 days) and adult (3 months) and not between cells from adult (3 months) and aged (30 months) rats. Moreover, a decline of the proliferative potential of satellite cells isolated from donors of increasing age has not been found in other studies[286,287]. Satellite cells isolated from adult human quadriceps muscles independent of age were always able to make between 15 and 20 divisions[157]. However, satellite cells from older donors took longer to grow out from the explants. Dodson and Allen[288] reported that the rate of proliferation in satellite cells did not appear to be depressed with advancing age, and they suggested that cells from old animals rather differ in the length of the lag phase of growth in culture and might therefore be less response to agents that activate them. Together, if there is a reduced proliferative potential in aged compared to adult satellite cells, it may be rather small. Nevertheless, reparative processes in severely injured muscles are markedly impaired in old rats compared to adult animals in regard to both the rate of regeneration and the amount of muscle replaced. Recovery after bupivacaine

injection or hindlimb unloading is slower and markedly impaired in aged rats[284,289]. However, a slight depletion of the satellite cell population combined with a minor reduction in its proliferative activity may not or not alone be accountable for the insufficiency of the reparative response in skeletal muscle of senescent animals.

Activation of satellite cells and differentiation into myotubes could also have an effect on the regeneration ability of aged muscles. Myoblasts derived from old satellite cells might not differentiate and fuse as well as those from young adult satellite cells[290]. Recently, it has been demonstrated that satellite cells isolated from skeletal muscle of aged rats exhibit decreased differentiation into myotubes compared with those isolated from young rats[291]. Conboy et al.[292] reported that the impaired regenerative potential in response to injury in aged mice is due to an impairment of activation rather than a decline in number of satellite cells. Quantification of the number of purified satellite cells isolated from hindlimb muscles of young (2-4 months) and old (23-24 months) mice by FACS (fluorescence-activated cell sorting) didn't reveal significant differences. Experimental modulation of Notch signaling, which plays a critical role in satellite cell activation and adult muscle regeneration[293] dramatically affected muscle regeneration in young and aged muscle. Inhibition of Notch signaling reduced the regenerative potential in response to injury in adult mouse muscles. Bromodeoxyuridine (BrdU) incorporation into nascent myotubes and formation of myotubes was reduced. Activation of Notch signaling, on the other hand, promoted BrdU incorporation and regeneration in old muscle to a level observed in adult muscle. Thus, the impaired regenerative potential of old muscles can be, at least partially, attributed to an impaired activation of satellite cells. More recently, this effect was confirmed in a model of parabiosis where young and old mice had a shared circulation[294]. Exposure of old muscle to the blood supply of a young mouse restored the regenerative potential that was normally impaired in senescence. The efficacy of muscle regeneration was increased without recruitment of young cells from the shared circulation. The authors concluded that the parabiosis restored the activation of Notch signaling and sequentially the activation of satellite cells in aged muscles. In summary, impaired activation of satellite cell in response to introduced injury appears to be partially accountable for the decreased reparative reaction of aged muscles.

Results obtained in cross-age transplantation experiments also demonstrated that there are sufficient cellular reserves to allow old muscles to regenerate as well as young ones if they are both placed in comparable environments. EDL muscles from 24-months-old rats were grafted into the bed of EDL muscles from 4-months-old hosts, and the EDL muscles from the young rats were transplanted into the EDL beds of the old rats. Measurements of *in vitro* contractile properties on the cross-age grafts as well as the same-age controls on the collateral limbs showed that the old-into-young grafts regenerated almost as well as young muscle autografts, whereas the young-into-old grafts regenerated no better than the old-into-old controls. Moreover, muscle mass and histological appearance was similar between old-into-young grafts and young autografts and between young-into-old grafts and old autografts, respectively[295,296]. Even the muscle of an old rat showing evidence of hind-limb neuropathy regenerated to almost control level after transplantation into a young host. These results suggest that old muscles have the intrinsic capacity to regenerate well and that the host environment in which the graft develops is the major determinant of the success of regeneration. Among the factors in the host environment that could account for the level of muscle fiber regeneration, innervation is important, because muscle grafts must become innervated by motor nerve fibers regenerating from host nerves. Further experiments have shown little difference in the success of muscle regeneration between young and old rats in either absence of innervation or the presence of full innervation[297]. Thus, it is likely that the success of skeletal muscle regeneration in old animals is, to a large extent, determined by the success of axonal regeneration of the nerve fibers that will ultimately supply the transplanted muscles or,

eventually, by the formation of the nerve–muscle contact. Even a reduced satellite cell population in aged animals is sufficient to repair a damaged muscle to near control levels of mass and force if conditions for regeneration are favorable, like for example muscle fiber damage without damage to the motor nerve supply or cross-age transplantation into young hosts[295]. Advanced age itself does not appear to limit the regenerative potential of skeletal muscle. Grounds[298] concluded that the overall regenerative capacity of satellite cells is not impaired in old muscles, despite the fact that the proliferative response of these cells becomes retarded with aging. However, the success of muscle fiber regeneration depends not only on adequate satellite cell population, but also on adequate innervation to complete the regenerative process[299].

Moreover, impaired regenerative response to introduced injury in aged animals does not necessarily mean that impaired regeneration is responsible for sarcopenia. In contrast, it has been recently shown that sarcopenia is not due to lack of regenerative drive[300]. The initial drive to regenerate myofibers, based on density of cell nuclei, occurrence of aberrant fibers, fibers expressing embryonic myosin, expression of myogenic regulatory factors, and level of IGF-1, is mostly marked in cases with the most advanced loss of muscle mass. Thus, the regenerative drive is not decreased, but increased in sarcopenia. This striking regenerative phenotype in sarcopenic skeletal muscle reflects a tissue in a state of continuous repair. BrdU incorporation experiments showed that age-associated loss of muscle mass is accompanied by increased myonuclear density that involves fusion of proliferative satellite cells[271]. This clearly implicates ongoing regeneration in aged muscles. The reaction to hindlimb suspension and consecutive reloading then showed an impaired regeneration in old compared with young animals. An increase in proliferative myofiber nuclei (incorporated satellite cells) was only observed in young animals, indicating impaired regeneration after unloading in aged animals. The aged muscle appears to already be in a state of regeneration and hence, does not react to injury cues that induce regeneration. However, that does not necessarily emphasize that impaired regeneration induces sarcopenia. Important may rather be the events that induce the regenerative phenotype in aged animals and prevent the regeneration from being successful.

Furthermore, it has been demonstrated that the difference in satellite cell number between young and old rats disappears after activation of the satellite cell by 2-month of denervation. The number of satellite cell was significantly increased and reached similar levels in both young and old animals after denervation[301]. Thus, aging does not repress the capacity of satellite cells to become activated and grow in response to denervation. In contrast to experiments in which the reparative response of satellite cells in aged muscle was initiated by the death of associated muscle fibers by, for example, bupivacaine injection, the response of satellite cells to denervation appears not to be reduced in age. Stimulation of satellite cell response by denervation may be more relevant for sarcopenia than introduced injury. Advanced age itself does not appear to be a key factor that limits the regeneration potential of satellite cells in senescent muscle under a condition in which reparative processes are triggered by muscle fiber denervation. Multiple lines of evidences suggest that sarcopenia represents such a condition (see below). Furthermore, sarcopenia is associated with an increase in muscle fiber heterogeneity, which depends on atrophy and hypertrophy of fibers[238]. Induction of skeletal muscle hypertrophy involves the addition of newly formed myonuclei via the fusion of satellite cells to the adult myofiber[302]. Irridation that causes reproductive death of satellite cells prevents compensatory hypertrophy in response to increased skeletal muscle loading[303,304]. Therefore, skeletal muscle hypertrophy in aged muscles exclude a general failure of satellite cell metabolism.

In summary, aging may influence the number of satellite cells and probably their initial response to an activating stimulus, but the intrinsic overall growth potential of the entire satellite cell population in response to denervation appears to remain similar in young and old skeletal muscle, regardless of their local and systemic environment. However, regeneration is not successful in aged skeletal

muscles, as the age-related loss of muscle mass can't be prevented. Impaired final maturation of regenerated myofibers into fully functional fibers could be responsible for the unsuccessful pattern of regeneration. Sarcopenic muscle seems not to be in a state of poor responsivness, but the aged motoneurons are less successful than adult motoneurons in innervating denervated muscle fibers[147]. In agreement with this hypothesis, myogenesis in long-term denervated muscle is abortive and does not lead to the formation of normal muscle fibers, despite activation of satellite cells[305]. Thus, innervation is of essential importance to form muscle fibers from activated satellite cells. Satellite cells from denervated muscles have been shown to undergo phenotypic modulation, including resistance to recruitment into the mitotic cycle and a robust extension of the nonproliferative compartment[306]. These characteristics of satellite cells deprived of neural influence may account for the inability of denervated muscle to fully regenerate.

1.4.5 Disruption or deterioration of the neuromuscular connection

Multiple lines of evidence indicate that changes in the neural activation of muscle significantly contribute to the progressive muscle weakness that is a hallmark of advancing age. Changes in the structural integrity of neuromuscular innervation, functional changes such as decreases in neurotransmitter release, and changes in motor unit size and other properties have been observed in many skeletal muscles in several animal species with advancing age. The cellular mechanisms underlying progressive structural and functional changes in neuromuscular innervation have remained elusive. Disruption of the neuromuscular connection can be caused either by loss of the innervating motoneuron or by deterioration of the NMJ. The loss of a motoneuron results in the denervation of the muscle fibers of the entire motor unit, whereas the deterioration of the NMJ only denervates an individual muscle fiber and may leave the other muscle fibers innervated by the same motoneuron intact. Investigations on motoneurons and NMJs indicate that both degeneration of motoneurons and deterioration of NMJs might contribute to age-associated denervation and subsequent muscle atrophy.

1.4.5.1 Age-related increase of denervated muscle fibers

The loss of muscle mass may be secondary to age-related denervation of muscle fibers. Denervation removes the trophic influence on the fiber and leads to atrophy. The mass of a muscle declines rapidly, falling to less than half its original weight within a month of denervation[307]. Experimental studies of total sciatic section in the rat have essentially contributed to the knowledge of the effects of long-term denervation of mammalian skeletal muscle[283,308,309]. The mass of the affected muscles falls rapidly within 5 to 7 days of denervation and declines further to 30-50% of control weight in succeeding weeks[310-312]. After several months, muscle weight remains constant at 5-20% of control[268,313]. Individual fibers show reduction of 70% in CSA over a period of months, and over 90% in the longer term[314,315]. Initially type II fibers are more susceptible to atrophy than type I fibers, but over prolonged periods the fibers atrophy to a similar extent[316-318]. This reduction in fiber CSA is accompanied by a progressive increase in interstitial collagen and fat. The number of myonuclei per muscle fiber is also declining in respond to denervation and may contribute to the shrinkage in thickness of the multinucleated muscle fiber. Interestingly, the number of muscle fibers appears not to be changed even after prolonged time of denervation of up to 7 months[283]. Contradictory to this finding, a decrease in the number of fibers after 9 month of denervation has also been reported[319]. However, the authors suggested that the fiber count was an underestimate due to the small size of many fibers, which made it difficult to identify them under the light microscope. Interestingly, electrical stimulation of denervated fibers restored the initial number of fibers. In several studies, necrosis and evidence of regeneration has been found after prolonged time of denervation up to

several months[268,313,319]. Thereby, newly formed fibers appear not to undergo complete maturation, possibly due to the absence of innervation. Ultrastructurally, atrophic muscle fibers exhbit evidence of disorganization, including loss or misalignment of sarcomeres, dissociation of the T system and sarcoplasmic reticulum, and alterations in the sarcomeric location of mitochondria[320,321]. Effects of denervation on the NMJ include a complete disappearance of nerve terminals in 24 hours[85]. Postsynaptic AChR aggregates, however, persists over a long period of time. After 10 weeks of denervation of the rat thyroarytenoid muscle 70.5% of AChR clusters were preserved. Moreover, denervation does not prevent the normal growth-related increase in AChR number, at least for short periods[322]. A number of studies reported a spread of AChRs over the muscle membrane after several days of denervation that resulted in an increase of the sensitivity of the muscle to ACh[323]. This denervation supersensitivity is due to an increase in extrajunctional receptors following increased AChR synthesis and not due to a relocation or dispersal of junctional receptors, since a decline in junctional AChRs has not been found[86]. Consequentially, electrical stimulation of denervated muscle can suppress the denervation-induced synthesis of extrajunctional AChRs[323,324]. Ultrastructurally, denervation changes included shallowing of the synaptic cleft and decrease in number of secondary folds, alterations that were completely reversible after reinnervation[325]. Corresponding to these morphological changes force-generating capacity diminishes in respond to denervation. Long-term denervated rat muscles develop only 10% of tetanic force of a normally innervated muscle. This loss is usually accompanied by a reduction in specific force that may reflect the disorganization at the ultrastructural level. In humans, chronic denervation of muscles as a result of injuries to spinal cord or peripheral nerves induced marked loss of muscle mass and CSA and increase in overlying fat[326]. Recently, the effect of denervation of the tibialis anterior muscle has been studied in rabbits[327]. Muscle morphology was examined at 10, 36 and 51 weeks after denervation by selective section of motor branches of the common peroneal nerve. Muscle mass and CSA were reduced by 50-60% and tetanic force by 75%, with no apparent reduction in specific force. The loss of mass was associated with atrophy of type II fibers and increase in fibrous and adipose connective tissue. The muscles denervated for 10, 36, or 51 weeks showed no significant differences. Evidence of necrosis or regeneration was not found and the total number of fibers stayed constant. In agreement with this observation, reinnervation of denervated muscles after nerve crush reconstitute to initial morphology of the muscle in regard to mass and fiber diameter[328]. The atrophied fibers are able to extend to their initial size after regaining motoneuronal input.

Together, denervation introduced muscle atrophy shares some features with sarcopenia, including loss of mass, strength and CSA, increase in intramuscular noncontractile tissues, selective type II atrophy and, eventually, regeneration. Thus, the pathogenic mechanism leading to sarcopenia may include denervation of muscle fibers. Interestingly, partial denervation more closely resembles the aging phenotype. Partial denervation of rat plantaris muscle resulted in precocious aging-like changes[329]. Compensatory hypertrophy, angulated fibers, type grouping and grouped atrophy, increased level of axonal sprouting and degenerating NMJs have been observed 6-12 months postsurgery. The possibility of reinnervation in partial denervated muscles appears to be essential to induce a sarcopenia-like phenotype. Therefore, reinnervation of denervated muscle fibers appears to be a process that essentially contributes to the characteristic histophathological phenotype of sarcopenia. In agreement with this finding, extensive denervation accelerates the development of sarcopenia.

Corresponding to a role of denervation in sarcopenia, the proportion of muscle fibers that are denervated at a given time has been demonstrated to be considerably increased in old skeletal muscles. The expression of the neural cell adhesion molecule (NCAM), a marker for denervated muscle fibers, was anlayzed on skeletal muscle cross-sections[208]. 8% of the muscle fibers of old

rats were in a denervated state. In contrast, young rats exhibited only 1% of the fibers of the same muscle to be denervated. These observations indicate that muscle denervation and reinnervation is a lifelong process that is enhanced at old age. About 15% of the observed deficit in specific force can be explained by the presence of non-innervated muscle fibers. Larkin et al.[257] similarly found an increase of NCAM positive fibers from 2% in 12-month old to 4% in 24-month-old rats. Additionally, they found a relative increase in type I fibers and fiber type grouping, indicatives of reinnervation processes. Interestingly, nerve-repair grafting resulted in similar changes than aging in regard to fiber type composition, grouping, and denervation. These similarities suggest that denervation and/or diminished reinnervation may be important factors in aging effects on muscle. Recently, Delbono and colleagues[330] assessed age-related denervation using a combination of electrophysiological and immunohistochemical methods to detect tetrodotoxin (TTX)-resistant sodium channels ($Na_v1.5$) in the flexor digitorum brevis muscles of young and old mice. The TTX dose-response curve showed three populations of fibers in senescent mice, one similar to fibers of young mice (TTX sensitive), another similar to fibers of experimentally denervated muscles (TTX resistant) and a third group characterized by an intermediate state between the former two. The three categories represented fully innervated, fully denervated and partially denervated muscle fibers respectively. Partially and fully denervated fibers added up to approximately 50 % of all fibers tested, a number that concurs with the percentage of fibers positive for the $Na_v1.5$ channel by specific immunostaining. The extent of denervated fibers is surprisingly high and suggests that age-dependent denervation has been underestimated in previous studies.
In summary, denervation of muscle fibers is clearly increased in senescence and contributes to the etiology of sarcopenia. However, the extent to which fibers are denervated and the time a fiber stays denervated until it's reinnervated or lost are poorly understood. Nevertheless, atrophy and loss of muscle fibers following loss of motoneuronal input in combination with impaired reinnervation is considered to be an important mechanism underlying sarcopenia. There are mainly three possibilities for a muscle fiber to get denervated: motor neuron death, irreparable damage to peripheral nerve axons, or deterioration of the NMJ.

1.4.5.2 Age-dependent reinnervation of denervated muscle fibers

The aging neuromuscular system is thought to undergo a continual process of reorganization as fibers become denervated and eventually reinnervated by adjacent motor nerves. Since motor unit innervation rations may increase five- to tenfold, this is a powerful compensatory mechanism[331,332]. Thompson and Jansen[333] for example partially denervated the soleus muscle of rats and found that the remaining motor units sprouted to about four times their average normal size. Sprouting occurs either from the nerve terminals (terminal sprouting) or from the first node of Ranvier (nodal sprouting) and is rapid, with significant recovery of function within weeks. Several studies addressed the extent to which collateral reinnervation is able to compensate for age-associated neural deficits. Einsiedel and Luff[334] demonstrated that senescent motoneurons are not able to substantially increase the size of their peripheral fields by extensive collateral reinnervation. Partial denervation of the gastrocnemius muscle of old rats followed by reinnervation in a recovery period did not result in the change of motor unit size. Furthermore, Pestronk et al.[335], using botulinum toxin to denervate fibers in the rat soleus muscle, found that although this resulted in significant terminal sprouting in the muscles of young rats, there was no significant change in aged rats. However, this does not reflect an age-associated inability to sustain neuronal growth. Sprouting was significantly increased in aged compared to young rats prior to any denervation procedure[336]. Although the sprouting response after partial denervation was decreased in aged animals, the total extent of sprouting was still higher in the aged group. Explanations for the reduced response in aged animals are a maximum limit to the amount of sprout growth for each motoneuron or,

alternatively, the latency of axonal sprouting that may be greater in aged than in younger animals. In agreement with the later explanation, myelinated fiber regeneration after sciatic nerve crush was substantially retarded in aged compared to adult mice[337]. The number and diameter of myelinated fibers, myelin area and thickness and Schwann cell cytoplasm area was significantly reduced after 2 and 4 weeks of regeneration in aged animals. At 8 weeks, however, most of the differences disappeared, although some deficiencies were maintained in myelin architecture and Schwann cell cytoplasm. A comparison of muscle endplate reinnervation that followed crush injury of the sciatic nerve between young adult and aged rats demonstrated that the re-establishment of normal single motor innervation was impaired in age[338]. Abnormal nerve bundles, immature nerve terminals, and multiple innervation were substantially increased in aged compared to young adult animals. Jacob and Robbins[339] showed that after partial denervation of mouse soleus muscle, postsynaptic sites were nearly or completely reoccupied at 60 days after denervation in young muscle. In old muscle, about 22% of former junctions remained denervated. At the same time, motor unit size expanded about 2.5 times in young and 2 times in old muscle. Nerve terminal volume per motoneuron increased 60-100% compared to control in young but only 20-25% in old muscle. These findings indicate the inability of the aged motoneurons to expand their field of innervation. The same authors[340] found that forced expansion of the motor unit in old mice, in contrast to young mice, resulted in physiological deficits in both regenerated and nondenervated NMJs of the expanded motor unit, and a persistent increase in latency of newly regenerated junctions. This finding indicates that the formation of new NMJs is impaired in age and could play a critical role in age-related difficulties to innervate regenerated fibers and to maintain large motor units. Later, impaired neuromuscular regeneration in senescence after sciatic nerve-crushing injury has been confirmed in rats[341,342]. Delayed arrival of Schwann cells and axons at the NMJ, drastic and long-lasting decline in the dimensions of terminal Schwann cells and AChR sites, low degree in spatial overlap between terminal Schwann cells and AChR sites, persistent aberrant changes, such as multiple innervation and terminal axon sprouting, together with poorly formed collateral innervation, nerve bundles, and NMJs were observed in aged animals. Also here, age-dependant regeneration inabilities have been attributed to impairments in the later regeneration phase, in which the regenerating axon extended into the NMJ. Recently, the influence of age on the response of the NMJ to nerve transection and repair was assessed[343]. After nerve injury, regeneration was delayed and incomplete in aged animals. At 4 weeks, 67% of the young animals had measurable compound motor action potentials (CMAPs), whereas none of the aged animals showed electrical evidence of regeneration. At 16 weeks, amplitude recovery was 65% of the contralateral reference in young, but only 36% in aged animals. Moreover, aged animals did not improve from 8 to 16 weeks post injury. The aged NMJs showed significant fragmentation and loss of area while the young NMJs remained relatively stable. Concomitantly, age impaired peak upregulation of AChRs and muscle regulatory factors. This impaired response of the NMJ to denervation may contribute to age-related neuromuscular degeneration by impairing full neuromuscular recovery after microdenervation.

In summary, the capacity of reinnervation is markedly diminished in aged subjects, resulting in an increasing number of permanently denervated muscle fibers. Impaired reinnervation in age has been attributed to sprouting deficits, reduced axon growth, impaired Schwann cell-axon interaction, reduced axonal transport or difficulties to establish functional NMJs. The fact that sprouting is frequent in aged animals and that aged animals can obviously sustain a substantial amount of neuronal growth does not speak in favor of sprouting or axonal growth deficits. The reason why sprouting finally does not lead to an increase in motor unit size in aged animals may rather reflect the inability to build functional NMJs. Alternatively, differences in size between aged and adult motor units may also explain the inability of aged motoneurons to react to nerve injury. It has been

shown that aged motor units consist of clearly more muscle fibers than adult motor units, most likely due to compensatory innervation of denervated muscle fibers (see below). There might be an overall limit of muscle fiber that a motoneuron can support. Aged units may be close to the limit, whereas, in adult motor units, there are still resources to support an enlargement.

1.4.5.3 Age-dependent loss of motor units

The concept of the motor unit was defined by Liddell and Sherrington[344] and consists of the motoneuron and all the muscle fibers innervated by that motoneuron. The number of fibers innervated by a particular motoneuron is referred to as innervation ratio, which can vary greatly within a single muscle and between different muscles. A motor unit innervating the soleus muscle of rats, for example, contains 58 muscle fibers, whereas a motor unit of the tibialis anterior muscle contains 127 muscle fibers[256]. Essentially, if a motoneuron is lost, a motor unit is lost. Both animal and human studies suggest that motor units are lost with age.

Very early electromyographic methods were applied on human muscles to estimate the number of motor units. The principle of these method is to measure the amplitudes of the muscle action potentials generated, firstly, by a single motor unit of average size and, secondly, by the whole muscle. Then, the number of motor units within the muscle can be determined by division. McComas et al.[345] found a constant number of motor units innervating the extensor digitorum brevis muscle of 41 subjects between 4 and 58 years of age. At this age, muscle fiber number had already begun to fall. Thus, factors other than denervation of entire motor units might be implicated in the initial loss of muscle fibers. After the age of 60, an age-related decrease in the number of active motor units was observed in several studies[346-349] (**Fig. 9**). Proband over 70 years of age had a motor unit count less than half that of the subjects under 40 years. Interestingly, the size of the remaining motor unit potential was often enlarged in the elderly subjects. However, the electromyographic studies may be influenced by age-related alteration of the signal transmission from nerve to muscle due to structural or functional changes of the NMJ. On morphological basis, Tomlinson and Irving[350] studied, postmortem, the lumbosacral spinal cords of 47 subjects between 13 and 95 years of age, and found that, although individual counts varied considerably in all age groups, there was no evidence of reduced number of motoneurons up to 60 years of age. Beyond 60 years, there were significant reductions in motoneuron numbers, with several cases exhibiting counts of only 50% of those found in young subjects, confirming the findings from electrophysiological studies. Based on a later electromyographic study using spike-triggered averaging, the estimated number of motor units in old (60 to 81 years) subjects was on average reduced to 53% of the number recorded in young (22 to 38 years) adults in the biceps brachii and brachialis muscles[145]. The motor units of the old subjects had, however, larger action potentials (23%). The association of a 23% increase in motor unit size and a 47% decrease in motor unit number indicates that motor unit loss is followed by incomplete compensatory reinnervation of denervated muscle fibers by adjacent motor units. Denervated muscle fibers not receiving compensatory reinnervation within a given time are atrophied and lost. An age-related increase in

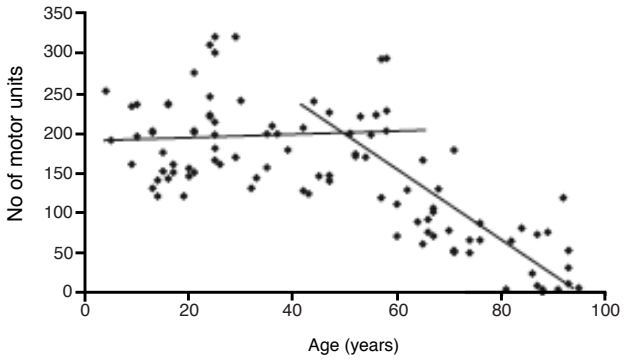


Figure 9 Relationship between the number of motor units in the extensor digitorum brevis muscle and the age of men between 5 and 88 years of age. The number of motor units remains constant from 5 to 50 years of age, but then decreases linearly with a zero intercept at 95 years of age. Modified by Faulkner et al.[236] from Campbell et al.[347]

motor unit size has also been found in the human thenar motor unit pool and was accompanied by a slowing of contraction speed. Such adaption may help to overcome age-related losses of motor units[351,352]. Recently, age-related decline in the motor unit number estimate (MUNE) has been confirmed in the tibialis anterior[353]. The MUNE was reduced from 150 in young (25 years) to 91 in old (65 years) and to 59 in very old (over 80 years) men. Despite the smaller MUNE at age 65, strength was not reduced in this age group, suggesting that age-related motor unit loss does not limit function until a critical threshold is reached. Compensatory enlargement of motor units could compensate the loss of motoneurons.

The muscles in the rat hindlimb have been studied extensively with regard to age-related changes in their motor unit populations[334]. Although some discrepancies are apparent in the findings, there is a general agreement among studies that substantial age related degeneration of the motoneurons is present in the senescent rat. Physiologic and morphometric studies have shown significant reductions of 40 to 75% in the estimated number of motor units in the hindlimb muscles[146,252,354,355]. However, direct determinations of the motoneuron number using retrograde labeling of motoneurons with horseradish peroxidase (HRP) revealed only a small decline in HRP-labeled alpha-motoneurons in the tibialis anterior but not in the soleus muscle of 135-weeks-old rats[234]. Burke and colleagues[144] counted motoneurons in an individual motor nucleus, the medial gastrocnemius (MG) motor nucleus. They found an age-associated reduction in the total number of motoneurons, which was due mainly to the loss of large-sized motoneurons, a finding that was confirmed in other studies[356,357]. The number of large motoneurons was reduced by 13% in the old (26 months) and by 30% in the very old (31 months) group and consequently, the mean average soma diameter was smaller in the older animals. Interestingly, a negative correlation between soma size and oxidative enzyme activity of motoneurons has been found[358], suggesting that the age-associated loss of large motoneurons may reflect a predominant loss of fast motoneurons with a low oxidative capacity. This could be an explanation for the predominant loss of fast, type II muscle fibers. Later, Hashizume and Kanda[359] reassessed the MG muscle motoneurons and found a significant decrease from an average of 132 in middle-aged rats to 121 in old rats (27 months), accompanied by a reduction in soma CSA of about 10%. Interestingly, there was no change in the number of motoneurons supplying the ulnar nerve in the rat forelimb. These findings suggest that the effects of aging on motoneurons and peripheral nerves innervating the MG muscle in the hindlimb are greater than those innervating forelimb muscles. The underlying mechanisms causing the differential extent and/or rate of progress of aging among different motor nuclei and peripheral nerves are not yet clear.

Morphological studies not only included the count of motoneuron cell bodies, but also the enumeration of myelinated fibers (MFs) in ventral spinal roots and peripheral nerves. An age-related decline has been shown in numerous reports in humans and rodents[252,354,360-365], but conflicting results exist[357,366-369]. In the soleus motor nerve of rats, the number of MFs decreased for example by 9 % with age[356]. In aged MG nerve a reduction in number (10%) and density (47%) of MFs has been found that was accompanied by increased fascicular area and fiber diameter, and myelin sheath irregularities[359]. Degenerative changes in myelin sheath structure, such as demyelination and remyelination, increase in amount of endoneuronal connective tissue, myelin infolding, and myelin reduplication have been found frequently both in ventral roots and peripheral nerve in aged humans and animals, regardless whether the actual number of MFs was reduced or not[357,360,367,370-373]. Degenerative changes in myelin sheath structure may reduce the conduction velocity in the peripheral nerves[374,375] and the spinal cord[376].

In concert with a reduced number of motor units with age, an enlargement of these motor units has been proposed from electrophysiological studies. Single motor unit action potentials were clearly increased in age, suggesting greater innervation ratios of aged motoneurons[145,346,347,377,378].

An age-related enlargement of motor units was confirmed on morphological basis using the glycogen depletion method[146]. Larsson and Ansved[256] reported that the motor units of the soleus muscle contained 58 and 84 muscle fibers in young (2 months) and old (20 months and older) rats, respectively. A motor unit of the tibialis anterior muscle consisted of 127 and 178 muscle fibers, in young and old animals, respectively, indicating a significant enlargement of motor units in age. Consequently, motor unit areas in the old animals were increased from 3.9 to 6.4 and from 8.8 to 12.5 mm^2 in soleus and tibialis anterior muscle, respectively. The loss of motor units may partially be compensated by the reinnervation of denervated muscle fibers by adjacent motor nerves, thereby increasing the number of innervated fibers per motor unit. The enlargement predominantly affected the low-threshold motor units in which the innervated muscle fibers belong to type I and was attributed to the sprouting of new collaterals from nerves innervating type I fibers in order to innervate part of the denervated type II fibers. This could be an explanation for the relative increase in type I fibers seen in aging rodents, since type II fibers which become reinnervated by type I motor unit axons actually become type I fibers with respect to biochemical and physiological properties[379]. Similarly, Kanda and Hashizume[380] reported a significant age-associated reduction in the mean tetanic tension of fast motor units, while the mean tension of slow motor units was substantially increased. An increase in the innervation ratio of slow motor units was the likely mechanism for the age-associated increases in their tetanic tensions. The reason for altered motor unit remodeling, in favor to slow motor units, is unclear; it might result from their superiority in establishing permanent connections with both type I and II muscle fibers, or from predominant loss of fast motoneurons. Whether motor units with specific physiological properties are lost in humans, as appears to be true for the rat, remains unclear.

The loss of large motoneurons, the associated remodeling of the motor unit, and the resulting electrophysiological changes were recently reexamined on the motor unit of the gastrocnemius muscle of aged compared to young and adult rats[381]. The decline in motoneuron number, preferentially among type II units, and associated evidence for motor unit remodeling started after 21/22 months of life and accelerated with advancing age. Important changes were found in the whole muscle tension, the specific tension (whole muscle tension per CSA), axonal conduction velocity and the CSA of type II fibers, which were all significantly reduced. Individual tension of type II motor units decreased, whereas tension of type I motor units continuously increased until the age of 36 months. Interestingly, the age-related change in tension produced by individual motor units was marginal compared to the decline in whole muscle tension. The maintenance of the motor unit tension indicated that the motor units had acquired additional muscle fibers, most likely recruited from those that were denervated due to death of their motoneuron. In some of these units, this process reduced or even exceeded a decrease in tension due to muscle fiber atrophy and a decrease in the specific tension. These observations, thus confirm that remodeling of the motor units in the aged individuals by recapturing of denervated fibers contributes substantially to the compensatory mechanism for age-related motoneuron loss.

Taken together, these results point towards an age associated reorganization of the motor unit pool, whereby selective losses or degradation of the fastest motor units are accompanied by increases in the proportion of slow motor units. The greater success of slow motor units in partially reinnervating muscle fibers, that have become denervated following the loss of their parent motoneurons, may possibly explain the apparent shifts in the fiber type composition in rat muscles. Previously described histological changes in the skeletal muscle, such as angular fibers and fiber-type grouping support the conclusion of ongoing remodeling of neuromuscular innervation in aged individuals[203].

In contrast to these findings, a couple of studies are available which do not confirm an age-related decrease in motor unit or motoneuron numbers. In an early report the number of cells in the

nucleus of the trochlear nerve in humans was determined in a total of 20 brains from newborn to 87 years of age. The cell count was 1830 in newborn, 2010 in the 87-year-old and 2115 in average over all the specimens examined[382]. The normal aging process was not accompanied by neural cell loss in this nucleus, which is in accordance with findings in five other brain stem nuclei examined by various investigators. Johnson et al.[383] did not find a significant decrease in the number of large motoneurons innervating the hindlimb muscles in aged (30 months old) rats with symptoms of hindlimb incapacities compared with adult (3 months old) rats. Nevertheless, a non-significant loss of about 15% of the motoneurons on cresyl violet-stained cross-sections was detected. An electromyographic assessment of the soleus motor units did not show a change in the estimated motor unit number between young (5-8 month) and old (25-30 month) mice[340], correlating with studies from rats[234] and humans[384] both finding the age-related change in soleus motor unit number to be minimal. Dalton et al.[384] explained the obvious discrepancy to earlier studies with the specific muscle and with methodological differences. The soleus muscle is a postural muscle, which is frequently activated. Since long-term physical exercises slow the age-related changes in motoneurons and peripheral nerves in rats[385], frequent activation of the soleus muscle could maintain motor axons and motor unit health. The contradiction to a further study, reporting 70% decrease in the MUNE of the soleus muscle[182] was attributed to the incremental stimulation technique, which is based on assumptions that may overestimates a MUNE, to the smaller sample size, and to the higher mean age of the subjects (about 90 years compared to 75 years). The dependence of age-related changes in motor unit estimates on the analyzed muscle was confirmed in a study on 79 human subjects between 20 and 98 years of age[386]. Motor unit populations were noted to decrease significantly with age in two distal muscles (thenar, extensor digitorum brevis) but appeared to remain constant in the biceps brachii. The excitable muscle mass, however, was diminished in all three muscles. The author concluded that muscle deterioration in the elderly is due to a combination of changes in the muscle fibers and in their nerve supply and that the extent may differ between proximal and distal muscles. In another study, the number, size, density and pathologic alterations of MF of ventral and dorsal roots and of peroneal and sural nerves in rats at 10, 20 and 30 months of age were analyzed[357]. Neither the number of MF per nerve nor the fascicular area were changed according to age. However, striking changes in MF size distribution, pathologic alterations of fibers and presence of regeneration clusters suggested age-related degenerative and regenerative events. These changes were most dramatic in the ventral root, where myelin infolding, myelin separation from axon and ballooning, as well as macrophagia and hyperplasia of Schwann cell nuclei were prominent. Importantly, axonal atrophy was observed in MF of the ventral root at old age. Morphometric changes of nerve fibers may complicate the counting of their number, and could lead to inexact or misinterpreted results. This might be a factor explaining the large discrepancies between MF estimates from different studies (see above). An extensive study on the total number of neurons in the fifth cervical and fourth lumbar dorsal root ganglion (containing afferent, sensory neurons) of 2- and 30-month old rats using stereological techniques and confocal microscopy revealed only a small decrease (12 %) in the number of neurons in the aged animals[387]. Moreover, a correlation between the degree of neuron loss and the extent of behavioral deficits among the aged individuals was not found. The authors concluded that loss of primary sensory neurons could not exclusively explain the functional deficits in sensory perception among senescent individuals. Although this study was not performed on motoneurons but on sensory neurons, the growing evidence that aging is not accompanied by a substantial loss of neurons[388-392] exclude a general instability and degeneration of neurons as an explanation for age-related motoneuron loss. A substantial loss of motoneurons (if it actually takes place) would have to be explained by different stabilities of afferent and efferent nerves. Furthermore, aging was not accompanied by neuron loss in most of the subcortical nuclei,

including the motor nucleus of the facial nerve, and the nuclei of the trochlear and abducens nerves[389].

It's important to keep in mind that several factors may limit the interpretation of studies on aging nervous system, including secular trends, species and strain differences, effects of tissue processing, and bias which may be introduced at many levels of an experimental design. Hippocampal cell death with aging has for example been generally accepted. Death of nerve cell in the hippocampus, a brain structure known to be critical to learning and memory, is a simple explanation for forgetfulness observed with aging and has been supported by a large number of studies. However, the development of more sophisticated and powerful methods of counting neurons have contradicted much of the early work[392,393]. It becomes now evident that neuron death is not an inevitable result of normal aging. A similar situation could also account for the impact and extent of age-related loss of motoneurons.

In summary, age-associated loss of motoneurons has been found in numerous studies, using a variety of morphological and physiological methods. Therefore, denervation because of neuronal death is one of the mechanisms that could account for atrophy and loss of some populations of muscle fibers. However, a subset of studies reported only small or no age-related change in motoneuron number. Especially motor units innervating the soleus may be less affected by age-related degeneration. Despite a minimal loss of motoneurons, age-associated muscle fiber loss has been found in a large number of reports on rat soleus muscle[146,196,197,199,201,233-235]. Therefore, the age-associated loss of muscle fibers may not be simply explainable by loss of motoneurons, at least not for all skeletal muscles. Generally, motor unit populations innervating different muscles undergo different age-related changes. Dramatic morphological alterations in the nerve fibers in regard to myelination or size complicate the analysis of nerve fibers and could influence the results. Furthermore, the variety of methods, subjects, and age-ranges makes it difficult to interpret the results. Especially indirect methods, like for example glycogen depletion or electromyographic studies could also be influenced by age-related changes at the NMJ or by age-associated events disconnecting excitation and muscle contraction, such as contraction-induced injury, age-related posttranslational modifications of contractile proteins, and excitation-contraction uncoupling (see above). Together, there is clear evidence for an age-related loss of motoneurons. However, the age of onset, the extent, and the influence on different muscles are unclear, making it difficult to estimate the impact of these motoneuron losses on muscle architecture and sarcopenia. There is increasing evidence that the loss of motoneurons is not the sole cause of the age-related muscle fiber loss. The loss of muscle fibers seems to exceed the loss of motoneurons. Moreover, the start of motoneuron loss occurs at high ages and may not precede the loss of muscle fibers (**Fig. 7, 9**). If senile muscle atrophy would be caused purely by death of motoneurons, it is difficult to explain why aging motoneuron have preserved cholinergic phenotype, and why they display no signs of cell-body atrophy[394,395]. It is also unclear whether the loss of motoneurons, if it actually takes place, is due to an inherent failure of the motoneuron or due to the inability or incapability of the muscle fiber to support the motoneuron, for example following deterioration of the NMJ. If there is an impairment of motoneuron function, the nature and origin of that impairment is largely unknown. Decline in neurotrophic factors and impairment of mitochondrial function due to oxidative damages[396] and increase in mitochondrial DNA mutations[397] have for example been suggested, but clear evidence has not been found.

Very early, it has alternatively been suggested that a slow loss of neuromuscular contact could be caused by degeneration of the endplate region, rather than by the loss of entire motoneurons. Gutmann and Hanzlikova[233] concluded that age-related fiber loss in rat soleus muscle, between adult (4 months) and old (24 months) animals, was secondary to a degeneration of motor endplates, which was found to cause a 33% decrease in motor unit innervation ratio but no change in the

number of motor units. Similarly the remodeling of motor units, i.e., the lifting of axon terminals of viable neurons from muscle fibers could be a mechanism that could account for the denervation of muscle fibers[95,398]. The amount of instability of the NMJ increases dramatically with advancing age, and muscle fibers that are rendered totally denervated during the remodeling process will undergo denervation atrophy until or unless they become reinnervated.

1.4.5.4 Age-related deterioration of the NMJ: cause or consequence of motoneuron loss?

The NMJ is the crucial link between motoneuron and muscle. Not alone signals for muscle contraction, but also trophic factors from nerve to muscle (anterograde signals) and vice versa (retrograde signals) pass via the NMJ. Therefore, the state of the NMJ is of outmost importance for the state of the motor unit. Deterioration of the NMJ could result via lacking or insufficient transmission of anterograde trophic signals in muscular deterioration and, conversely, via lacking or insufficient transmission of retrograde trophic signals in motoneuron deterioration. However, if not all NMJs of a motor unit are affected, deterioration of the motoneuron may not necessarily take place. Normally, the NMJ has a high safety factor, so that the arrival of an action potential at the motoneuron almost invariably results in an action potential in the muscle fiber. Consequently, any change in the security of synaptic transmission at the NMJ may profoundly affect the extent to which individual muscle fibers are recruited for normal function.

The endplate undergoes continual growth and degeneration in normal muscle of healthy adult mammels with gradual changes in pre- and postsynaptic components through adult life[399]. With advancing age the NMJ undergoes degenerative structural and functional changes. The Pretzel-like shape of the NMJ typically found in the young adult is remodeled and becomes increasingly fragmented and dispersed with advancing age, resulting in more distinct synaptic regions. The fragmentation occurs on both the presynaptic and the postsynaptic side. It is reflected by the distribution of AChR clusters[233] and AChE[399], and by the structure of presynaptic nerve terminals[400,401]. Courtney and Steinbach[92] labeled postsynaptic AchRs with a fluorescent derivative of α-bungarotoxin and found an increase in the number of discrete regions of high AChR density in junctions from older rats. In another study, the percentage of fragmented NMJs, visulaized by cholinesterase stainings, gradually increased with age. NMJs classified as normal completely disappeared in rats older than 15 months. The gradual and progressive loss of synaptic contacts resulted in the loss of entire NMJs[95]. Prakash and Sieck[402] counted the number of nerve terminal and motor endplate branches on type-identified diaphragm muscle fibers and found a significant increase between 6-month-old and 24-month-old rats on all fiber types, indicating age-related fragmentation of pre- and postsynaptic sites. Thereby, the aging associated fragmentation was found to be more severe on type II than on type I muscle fibers. This finding could be an explanation for the age-associated increase in proportion of type I fibers seen in rodents. The capability to support the associated fibers may be greater among the less fragmented and thus, more stable NMJs on type I fibers. In an electron microscopy based morphological comparison of NMJs on the gastrocnemius muscle of the mouse, only 40% of the NMJ in old mice were classified as normal, compared to 85% in young mice[403]. In several studies presynaptic nerve endings were analyzed by zinc iodide-osmium stainings in adult and aged mice. Increased numbers of nerve terminal regions were found on soleus, EDL[401] and masseter muscles[400] of old (30 months) compared to adult (5-6 months) mice. The increase in the number of presynaptic regions was associated with a decrease in size per region, reflecting an ongoing dispersal of the nerve terminal with age. It has been speculated that this redistribution is due to constriction and retractions of nerve terminals in old mice, which may result from loss of adhesion of nerve terminals to the synaptic matrix or surrounding Schwann cells[404]. It is possible that a component of the adhesion complex could be defective in old mice, resulting in diminished integrity of the entire complex. For instance, in old

mice the complex might be affected by reduced axonal transport of cytoskeletal or membrane components, altered synaptic matrix due to proteases, or primary changes in muscle or Schwann cell.

A number of studies tried to analyze age-related alterations in the size of both pre- and postsynaptic parts of the NMJ. Deschenes and Wilson[405] found a drop of 12 and 14% in the stained area of antisynapsin labeled presynapses and α-bungarotoxin labeled postsynapses, respectively, in aged compared to young adult rats. Apel et al.[343], in contrast, did not detect any significant differences between α-bungarotoxin labeled postsynaptic areas of young compared to aged NMJs, a finding that was confirmed in another study using cholinesterase staining[406]. Robbins and Fahim[401], finally, found a tendency for age-related increase in zinc-iodide stained nerve terminal area. Analysis of nerve terminal area on type-identified diaphragm muscle fiber revealed fiber type specific differences. The area of aged endplates was increased on type IIx and type IIb but not on type I and type IIa fibers[402]. Moreover, extensive analysis of endplate areas on different rat muscles revealed substantial muscle specific differences[93]. Together, fiber type and muscle dependent variations as well as methodological distinctions may be explanations for the diversity of results obtained in different studies. Methodological difficulties include the analysis of NMJs on single muscle fibers, where it is not always positioned on top but can be wrapped around the fiber. Quantification of tilted NMJs leads to underestimation of the areas. NMJ size is also dependent on fiber size and has been shown to progressively increase over an animal's life[83]. Thus, to judge age-dependent differences, not only the age of the animals categorized as "old" but also the age of the "young" animals may be important. The progressive growth of the NMJ was substantial in the first months of a mouse life and slowed down during later adulthood. Whether this trend is reversed at a certain age or just slowly continues is not fully clear, but if there are changes in senescence, they may be rather small.

Further structural alterations of the NMJs of old mice included degenerative features such as partially innervated and vacated endplates, abnormalities of junctional folds, degenerations of Schwann cells, extra-junctional AChR, and increased length and complexity of the endplate[92,399,403]. Furthermore, decline in synaptic vesicle density[403,407,408] has been reported. The final stage of the disturbance of the NMJ is the appearance of "silent" axons, characterized by retraction of axonal terminals and disruption of the postsynaptic membrane[409]. At the same time, evidence for regenerative processes, such as terminal sprouting and multiple innervation have also been observed[336,410,411]. It has been suggested that two major processes account for morphological alterations in NMJs of mature rodents: changes that are simple continuations of normal development and late changes that reflect newly arising age-dependent extrinsic or intrinsic factors. Interestingly, it has been shown that aged NMJs underwent significant remodeling of both pre- and postsynaptic parts following muscle unloading, whereas adult NMJs maintained normal morphological characteristics[405]. Similarly, aged NMJ exhibited significant fragmentation and loss of motor endplate area after nerve injury and repair while the young NMJ remained relatively stable[343]. Aged NMJs may be generally less stable than young ones, rendering them more susceptible to disturbances such as unloading or denervation.

Only few studies on NMJ morphology in age were performed on human muscles. However, the situation appears to be similar. Oda[399] used human intercostal muscle fibers and showed a greater number of smaller AChR conglomerates per endplate in older subjects. Additionally, perijunctional AChRs were observed and the number of preterminal axons entering an endplate was increased.

In recent years, Lichtman and others have developed vital stainings and video microscopy techniques that have allowed the same NMJ to be monitored over time in living animals[81]. This method generates direct information about the life history of NMJs and sheds light on structural changes in the development and aging of synapses. The pre- and postsynaptic components can be

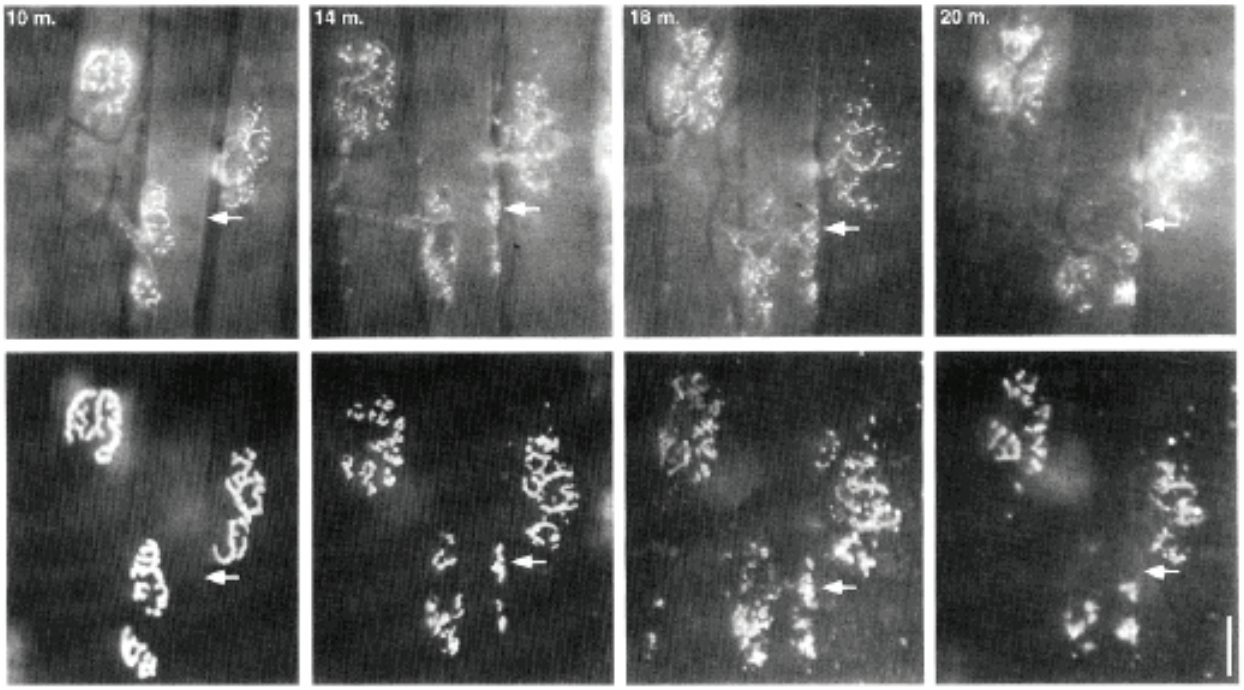


Figure 10 Age-related changes in the stability of the NMJs. Vital fluorescent dyes and video microscopy were used to follow NMJs in the mouse sternomastoid muscle over time. Motor nerve terminals (top row) and postsynaptic AChRs (bottom row) were stained. After digital images were obtained, the animal was allowed to recover. Several months later, the experiment was repeated and the same junctions were re-located and re-imaged. Three junctions viewed at 10, 14, 18 and 20 months of age are shown. Age-related changes include loss of existing synaptic areas (compare 10 m and 14 m panels), compensatory addition of new synaptic sites (arrow in 14 m panel), further loss and addition in these areas (18 m panel) followed by almost complete disintegration of the junction (20 m panel). Scale bar, 20 μm. From Balice-Gordon[148].

vitally stained with fluorescent dyes and imaged, without inducing damage. Using these *in vivo* imaging approaches, the growth, maintenance and aging of NMJs in the sternomastoid muscle of mice has been studied. Following the developmental period of synapse elimination, NMJs are remarkably stable in the pattern of presynaptic motor nerve terminals and postsynaptic receptor rich areas. For the most part, the NMJs grow by expansion of existing elements without the addition or loss of synaptic regions[83]. In mice older than 12-15 months of age, however, the stability of neuromuscular innervation decreases, a process becoming more marked beyond 18-20 months of age. The aging process has extensively been studied by Balice-Gordon[148] using the sternomastoid muscle of rats (**Fig. 10**). The same NMJs were continuously imaged between 10 and 36 months of age. At early times (12-14 months of age), only a small percentage of the NMJs undergo loss of motor nerve terminal branches and underlying receptor rich areas. The loss of postsynaptic AChR areas occurs via the gradual loss of receptors from a region, followed by loss of the overlying nerve terminal. Pre- and postsynaptic loss occurs one region at a time rather than all at once. This sequence of events is strikingly similar to that observed during naturally occurring synapse elimination during development[412] and synapse elimination following reinnervation[90]. Thus, it is possible that the cellular mechanisms underlying synaptic loss may be similar in development, reinnervation and aging. At 12-18 months of age, in some junctions, motor neurons appear to compensate for small losses of synaptic area by sprouting and adding new sites. The proportion of NMJs undergoing loss and addition of synaptic sites increases over time. However, the incidence of compensatory addition decreases dramatically with age. Moreover, the newly formed synaptic sites also appear to be unstable, with many disappearing within several weeks of their formation, which is a marked contrast to the stability of NMJs at earlier ages. By 24-36 months of age,

the vast majority of NMJs have undergone significant losses of pre- and postsynaptic sites. In some cases these structural alterations were associated with degenerative changes of postsynaptic muscle fibers themselves. At 36 months loss of existing synaptic areas and compensatory addition of new synaptic sites is followed by almost complete disintegration of the junction.

Beside structural alterations with age, there is also evidence for functional impairment. The ability to sustain synaptic transmission at the NMJ is diminished with advancing age[413-415]. Banker at al.[407] analyzed neuromuscular transmission in different muscles of young (8-12 months) and old (29-33 months) mice. The frequency of spontaneous miniature endplate potentials was decreased with age in the majority of the muscles but their amplitudes were unchanged. Evoked endplate potential amplitudes, however, were increased by about 100%. This suggested that the number of quanta released per nerve impulse is increased with age, a finding that was confirmed by direct measurements[414,416]. This may rather reflect a compensatory than a degenerative response. Motor inactivity is an obvious candidate for the stimulus for this compensation, especially since synaptic efficacy increases after experimental disuse in young animals[417,418]. Lower level of AChRs may be partially compensated by increased transmitter release. The age-related increase in quantal content could be accounted for by an increase in numbers of synaptic vesicles per terminal, active zones per terminal or terminals per endplate. Utrastructural morphometric analysis showed age-related loss of synaptic vesicles in the majority of reports[403,407,408], but the opposite situation has also been found[406]. Increase in the number of nerve terminals per endplate, and by inference the number of release sites, has been observed constantly[93,401,415]. Total neurotransmitter level at the NMJ, however, has been shown to decrease with age, probably due to increased quantal content after nerve stimulation and increased leakage in the resting state[414]. Thus, the fraction of transmitter that is released per action potential must increase in age, leading to an enhanced synaptic depression in aged animals. Consequentially, the rate of transmitter turnover in old mice is increased[419]. However, in more advanced ages the demands of increased transmitter turnover might become overwhelming and the ability to sustain synaptic transmission is decreased. Observations of NMJ function evaluated with intracellular recordings of nerve evoked endplate potentials in muscle fibers of aged mice (24-36 months of age) *in vitro* suggest that at most junctions, synaptic transmission fails more than half of the time to activate muscle fibers when the muscle nerve is stimulated at relatively low frequencies (10 to 20 Hz). At these and higher frequencies, endplate potentials fall below threshold for initiation of a muscle fiber action potential and thus activation of muscle fiber contraction.

Progressive changes in the presynaptic populations of motoneurons, in the postsynaptic population of muscle fibers, or at the NMJ itself are likely to contribute to the synaptic instability observed as animal age. Additionally, Schwann cells have been shown to play an important role in axon outgrowth and synaptic repair[420] and clearly show age-related abnormalities[341,342]. Together, age-related changes in synaptic maintenance may be an adaption to changes in the number and viability of motoneurons, muscle fibers and Schwann cells or in the interactions among them. Loss of presynaptic motoneurons may lead to transient denervation of muscle fiber in aging animals, with subsequent reinnervation by sprouts from remaining motor axons that may become compromised in their ability to maintain excess synapses. In very aged animals decline in motoneuron number has been well documented, but there are conflicting reports about the onset and extent of motoneuron death (see above). As motor neurons die, NMJs might be expected to be dismantled abruptly rather than gradually. Contradictory, changes in synaptic maintenance occurred via a protracted time course[148]. Alternatively, before the final event of cell death, motoneurons may gradually lose their ability to maintain all their endings, resulting in the loss of one synaptic site after another. However, the sequence of events, gradual loss of postsynaptic receptors followed by loss of overlying nerve terminals, does not speak in favor of a denervation due to motoneuronal death.

On the other hand, a number of observations argue that the postsynaptic muscle fiber also plays an important role in synaptic maintenance. Muscle fibers in aged animals become progressively atrophic, and may become more susceptible to damage during normal behavior than muscle from young adult animals. Increased muscle degeneration in aging animals may induce synaptic loss on compromised fibers in advance of their removal from the muscle. Death of postsynaptic muscle fibers has been shown to lead to the loss of synaptic sites on residual basal lamina ghosts in young adult mice[90] and to cause the sprouting of motor nerve terminals innervating adjacent intact muscle fibers. This and other work suggest that healthy postsynaptic muscle fibers provide essential factors for normal synaptic maintenance. Trophic factors have been shown to play key roles in motoneuron survival, nerve –muscle maturation and in synaptic maintenance[421]. Muscle derived trophic factors acting on motoneurons (neurotrophic factors) mediate developmental events such as naturally occurring cell death and promote maturation of NMJs. Similarly, motoneuron derived trophic factor acting on muscle fibers (myotrophic factors) may play a role in the generation of secondary myotubes and the maturation of fiber type characteristics in developing muscle. The identity of trophic factors has not been determined with certainty, although a number of candidate molecules have been proposed. Deterioration of the NMJ prevents the exchange of trophic factors, and hence, may induce degeneration of both motor axon and muscle fiber.
Together, an age-associated deterioration of the structure and the function of the NMJ takes place, ultimately causing structural and functional denervation. Denervated muscle fibers not receiving compensatory reinnervation within weeks become progressively atrophic and eventually disappear. Difficulties to establish functional NMJs could have influence on the regeneration of the muscle by reinnervation of denervated fibers. Newly formed muscle fibers can't be maintained if the formation of a stable NMJ is not possible. Regeneration has clearly been shown to be impaired in senescence and failure of reinnervation is one of the most convincing explanations. Whether a loss of motoneurons or muscle fibers induces the NMJ deterioration or whether a deterioration of the NMJ leads to the absence of trophic factor supply and consequentially death of the motoneuron and muscle fiber is currently not known.

1.4.6 Oxidative damages to mitochondria compromise mitochondrial function in age

One of the most popular mechanisms thought to cause aging in skeletal muscle and other highly metabolic active cells, such as neurons and cardiac myocytes, is oxidative stress. Reactive oxygen species (ROS), normally produced by mitochondria, may cause oxidative damage to organelles, lipids, proteins, and DNA (in particular mitochondrial DNA (mtDNA))[422]. Over a lifetime, the accumulated damage becomes sufficient to compromise cellular function and may result in permanent removal of the cell by apoptosis. This mechanism is consistent with the distribution of aging effects in skeletal muscle. Within the same muscle, myofibers can be differentially affected, suggesting a mechanism that distinguishes between distinct fibers and does not damage the whole muscle[423]. Mitochondrial production of ROS significantly increases in skeletal muscle over the course of aging[424]. ROS production was 1.7 fold greater in mitochondria isolated from 36-month compared to 6-month rats, and was accompanied by an increase in a DNA repair enzyme (8-oxoguanine glycosylase 1). ROS induced oxidative damage leads, over time, to mtDNA mutation deletions that result in dysfunctional mitochondria[425]. There are several characteristics of mtDNA that suggest it could be involved in the decline of mitochondrial function with age[426]. It codes for 13 subunits of the respiratory chain and oxidative phosphorylation system, 2 rRNAs and all the tRNAs necessary for translation. In detail, 7 subunits of complex I, 1 subunit of complex III, 3 subunits of complex IV, and 2 subunits of the ATP synthase are encoded by mtDNA. MtDNA is situated in the mitochondrial matrix where it may be vulnerable to damage from ROS generated

by the mitochondrial electron transport chain (ETC). The absence of DNA-protecting proteins like histones and some of the repair mechanisms associated with nuclear DNA renders mtDNA particularly susceptible to damage. Moreover, mtDNA undergoes a continuous turnover both in mitotic and post-mitotic cells and the DNA polimerase that is involved in mtDNA replication is believed to have a high error rate. This vulnerability of mtDNA has led to the suggestion that the accumulation of somatic mtDNA mutations might play a role in the aging process, especially in highly metabolic cells such as skeletal muscle. The preferential accumulation of deleterious mitochondrial mutations in a restricted subset of aging tissues may correlate with age-related deficiencies of function in those tissues.

Age-related decline in whole muscle oxidative capacity is well established[427-430], and was partially accounted for by reduced mitochondrial volume. Reduced volume of each mitochondria rather than a reduced number of mitochondria seems to be responsible for this age-associated loss of mitochondrial volume. Several studies constantly reported a loss of volume per mitochondria in the range of about 30%, whereas the results about the number of mitochondria are conflicting[431-433]. Reduced mitochondrial volume is also reflected by a decline in the number of copies of mtDNA and the concentration of mtDNA transcripts in older muscles[434,435].

Interestingly, Conley et al.[428] found that not only reduced mitochondrial volume density but also reduced oxidative capacity per mitochondrial volume were responsible for the loss of oxidative capacity per volume of vastus lateralis muscle seen in elderly compared to adult human subjects. Reduced capacity of the mitochondria themselves implies mitochondrial dysfunction with age. Indeed, a number of studies found a progressive age-associated decline in mitochondrial respiratory chain function in skeletal muscle mitochondria[422,426,436-442]. Especially complexes I and IV of the ETC and the ATP synthase are susceptible to age-related alterations, whereas complexes II and III are largely unaffected. This reflects the extent to which these complexes are encoded by mtDNA, indicating an influence of age-associated mitochondrial DNA mutations on ETC function. Cooper et al.[426], using human skeletal muscle mitochondria, showed that the activities of complexes I and IV decreased by 59% and 47%, respectively between 20-30 years and 60-90 years of age. Substantial reductions in ETC functions occurred also in mitochondria harvested from the gastrocnemius muscle of old mice (20 months) compared to those from adult mice (10 months)[437]. Most notable were reductions in the activities of complexes I, III, and IV, kinetic alterations for complexes III and IV, and loss of high-affinity sites for complex IV. Complex II, the one complex without mtDNA-coded component, was not affected. Another indication for age-associated mitochondrial dysfunction is the disproportionate change in one enzyme versus another that can be observed in aged skeletal muscle. Hagen et al.[425] demonstrated that the reduction in flux through ETS complexes I-III was greater than the reduction in citrate synthase activity in older rats. Moreover, it has been shown that mitochondrial abnormalities are more frequent in muscles undergoing sarcopenia[153]. The number of muscle fibers with ETC abnormalities, represented by complex IV (cytochrome-c oxidase, COX) deficiencies and increase in complex II (succinate dehydrogenase, SDH) activity, was increased with age in vastus lateralis and soleus muscles of rats. Fiber reconstructions showed that the cross-sectional area of ETC-abnormal fibers decreased in the abnormal region (region displaying COX-/SDH+ phenotype), whereas control fibers did not. Muscles undergoing high degree of sarcopenia in regard to fiber loss, exhibited more ETC abnormalities, suggesting a direct association between ETC abnormalities and fiber loss. Wanagat et al.[152] found that ETC abnormalities are increased from 289 in 5-months-old rats to 1094 in 38-months-old rats in the entire rectus femoris muscle. Additionally, segmental mitochondrial abnormalities contained mitochondrial DNA mutations as revealed by laser capture microdissection and whole mitochondrial genome amplification. Muscle fibers harboring mitochondria deletions often displayed atrophy and splitting. It has been concluded that age-associated mitochondrial

DNA mutations play a causal role in sarcopenia.
Mitochondrial DNA mutations have long been thought to compromise mitochondrial function[443]. Small deletions and insertions[444,445], as well as large deletions[446,447] and specific point mutations[448] have been observed in mtDNA of diverse tissues with aging. Cortopassi and Arnheim[449] probed mtDNA from older humans for a specific 5 kb deletion previously found only in patients affected with the rare neuromuscular diseases Kearns-Sayre syndrome and progressive external opthalmoplegia. They found this deletion in heart and brain of older adults, but failed to detect it in fetal tissues, suggesting that this pathogenic deletion could accumulate with age. Indeed, in old skeletal muscle, accumulation of this 5kb deletion of mtDNA was found in several studies[426,440,450,451]. The age-associated accumulation of this mutation and ETC deficiency appear to be chronologically and quantitatively correlated. However, the low absolute level of this mutation on older subjects is unlikely to contribute significantly to the observed mitochondrial dysfunction. The increased incidence of the 5kb deletion could rather be indicative of multiple accompanying mtDNA mutations, and the decrease in ETC activities could be the result of an age-associated increase in random mtDNA mutations. Torii et al.[452], examining diaphragm muscle from 34 humans, detected multiple mtDNA deletions particularly among the elderly. A 3.4 kb deletion of mtDNA was found in 0% of the subjects under age 30, in 28.6% of those in their sixties, in 72.7 % of those in their seventies and in all of those over age 80. This deletion included the genes for four subunits of complexes I and IV. The activities of these two complexes have been shown to decrease most dramatically in relation to age, suggesting a direct connection between mutation and functional deficits. Hayakawa et al.[453,454] more directly showed that ROS were responsible for the age-associated accumulations of mtDNA mutations. A hydroxyl-radical adduct of deoxyguanosine (dG), 8-Hydroxy-deoxyguanosine (8-OH-dG), accumulated in mtDNA from human diaphragm and heart muscles. In subjects below 55 years of age, the level of 8-OH-dG in diaphragm mtDNA was below 0.05% of the total dG, whereas, in subjects over 65 years of age, the level of 8-OH-dG increased with age at a rate of 0.25% per 10 years, reaching 0.51% at age 85. In addition, a concomitant increase in multiple deletions was detected with increasing age. Accumulations of 8-OH-dG in mtDNA could trigger double-strand separation that favors DNA deletions.
The percentage of mtDNA mutations accumulating with age varies substantially from tissue to tissue. Selective amplification of the common 5kb deletion of the mitochondrial genome has been used to quantify the extent of the deletion's accumulation in a variety of human tissues, including heart, brainstem, brain cortex, psoas and diaphragm muscle, liver, kidney, skin, spleen, testis, and lung[450,455,456]. The deletion occurred at much higher level in nervous and muscle tissues than in all other tissues studied. Skeletal muscle and nervous tissue are especially susceptible for mtDNA mutations with age, a finding that could be accounted for by the high metabolic rate of these tissues, producing high amounts of ROS.
Not only mtDNA but also mitochondrial proteins and lipids are a target for oxidation by ROS. Oxidative modifications of proteins increase with age and lead to protein cross-linking, proteolysis and loss of functional activity[457]. Choksi et al.[458] found for example that a dramatic age-related decline in almost all ETC complex activities correlates with increased oxidative modification of the ETC subunits, whereas the overall protein amount was not changed. Oxidative damages to ETC subunits by mitochondrial ROS may lead to further mitochondrial dysfunction. Enhanced lipid peroxidation, on the other hand, leads to decreased fluidity of cell membranes[459]. Oxidative damage of cardiolipin, a crucial component of the inner mitochondrial membrane, is particularly deleterious as this phospholipid is required for the activity of mitochondrial enzymes.
Very recently, it has been shown that mice lacking the antioxidant enzyme CuZnSOD (Sod1) are characterized by high levels of oxidative damage and an acceleration of sarcopenia[460]. Muscle atrophy in Sod1 deficient mice was accompanied by a progressive decline in bioenergetic function

and an elevation of mitochondrial ROS generation. As a consequence, more rapid induction of mitochondrial-mediated apoptosis and loss of myonuclei have been observed. Interestingly, aged Sod1-deficient mice showed increased NMJ fragmentation and denervation and the contractile force in these mice was greatly diminished. These findings demonstrate that increased ROS levels can lead to oxidative damage, mitochondrial dysfunction, muscle atrophy and even denervation and fragmentation of the NMJ.
Together, oxidative damages caused by ROS have an influence on aging skeletal muscle. The susceptibility of skeletal muscle and nervous tissue to ROS induced mtDNA damage and the prevalence of mitochondrial dysfunction in aged muscle provide evidence that this process contributes to the pathogenic ensemble resulting in sarcopenia. It is well-established that oxidative damages comprise the oxidative capacity of skeletal muscle. However, it is not fully understood whether and how oxidative damages can lead to muscle fiber atrophy and loss. There is increasing evidence that cell death by apoptosis could be involved.

1.4.7 Role of apoptosis in sarcopenia

Apoptosis denotes a form of cell death that is highly regulated and characterized by specific morphological, biochemical, and molecular events. It is essential to normal development of multicellular organisms and is involved in cell turnover and remodeling in healthy and diseased tissue. During apoptotic cell death, cells successively shrink without detectable release of their constituents. The shrunken apoptotic remnants are then phagocytosed by macrophages and their constituents recycled. Apoptosis is promoted by death-inducing signals, such as ROS and the cytokine tumor necrosis factor (TNF)-α, imbalances in calcium regulation, and alteration in the composition and abundance of Bcl-2 family proteins (Bax, Bad, Bcal-2, Bcl-xl, etc.). After this induction phase, nuclear activators, activation of cell surface receptors, or mitochondrial pathways, such as the release of cytochrome c and/or apoptosis inducing factor (AIF), contribute to the commitment to cellular death[461]. Cytoplasmic and nuclear events are involved in the execution of cell death. A cascade of protein-cleaving enzymes called caspases is activated in the cytosol and is responsible for the breakdown of a broad spectrum of cellular targets. In the nucleus, DNA fragmentation caused by activated endonucleases, chromatin condensation, and breakdown of the nuclear envelope occurs. There are also caspase-independent pathways, such as the release of AIF and endonuclease G (EndoG) from mitochondria, which induce large scale DNA fragmentation and apoptosis after translocation in the nucleus.
Accelerated apoptosis with aging has been found in several mitotic tissues, like for example liver and white blood cells, and may serve to prevent age-associated tumorgenesis. Apoptosis in post-mitotic tissues, such as skeletal muscle, in contrast, may result in diminished tissue function with normal aging. There is some evidence indicating a role of apoptosis in pathophysiological skeletal muscle cell loss. However, with respect to apoptosis, skeletal muscle is a unique tissue, because muscle cells are multinucleated. Within the individual muscle fiber, not all nuclei are transcriptionally equivalent, and each nucleus governs the surrounding cytoplasmic area, called the myonuclear domain[302]. Although muscle size can vary considerably under different conditions, the size of the myonuclear domain remains relatively stable, implying a fairly strict regulation of myonuclear number at least in muscles from young adults. This regulation is governed by two opposing processes: the gain of myonuclei by fusion of satellite cells into hypertrophying muscle fibers and the loss of nuclei in atrophying muscle fiber[461]. A decline in myonuclear number has been found in muscles undergoing atrophy in a variety of experimental conditions, such as spinal cord isolation and transection, microgravity, hind limb suspension and chronic denervation[271,462-469]. The process by which nuclei are eliminated from multinucleated muscle fibers shares similarities with

apoptosis, since it involves chromatin condensation and DNA fragmentation, which are considered to be hallmarks of apoptosis. However, apoptosis in a multinucleated myofiber should actually be termed apoptotic nuclear death because destruction of the entire cell does not necessarily follow elimination of the nucleus as in mononucleated cells[461]. There is some evidence that one or more myonuclei can be eliminated by apoptosis without affecting the other nuclei within the same cytoplasm. Therefore, the mechanisms by which nuclear cell death occurs in muscle fibers may be distinct from those involved in apoptosis in mononucleated cells where nuclear as well as cytoplasmic contents are cleared. Nevertheless, apoptosis plays an important role in skeletal muscle atrophy since it is increased in a number of pathological and physiological conditions. Motoneuron diseases, skeletal muscle denervation, spinal cord injury, muscular dystrophy, hindlimb suspension or immobilization, and acute exercise are all associated with an increase in DNA fragmentation that is usually associated with apoptosis[462,463,466,469-473].

The incidence of apoptosis in skeletal muscle with age has not been well investigated. Dirks and Leeuwenburgh[474] quantified apoptosis in 6-month-old and 24-month-old rats and found a 50% increase in cytosolic mononucleosomes and olignucleosomes in the aged compared with the young animals. Other apoptotic markers, such as cytosolic cytochrome c, caspase-3 activity, and mitochondrial Bcl-2 and Bax, were not altered with age. This finding does not favor the hypothesis that, with aging, mitochondrial dysfunction and oxidant stress to mitochondria could induce the mitochondrial permeability transition, the release of cytochrome-c, and subsequent initiation of apoptosis[475]. More recently, myocyte expression of procaspase-3, cleaved caspase-3 and the extent of DNA fragmentation were reported to be elevated in the gastrocnemius muscle of aged rats, suggesting an involvement of apoptosis-like processes in sarcopenia. However, activity of caspase-3 and levels of cytochrome c and caspase-9 were not changed with age[476]. The protein levels of XIAP, an inhibitor of caspase-3 were increased in the aged muscle, suggesting that a compensatory mechanism opposing the increase in caspase-3 protein content may dampen caspase-3 activity. Later, Leeuwenburgh et al.[477] found that EndoG co-localized with nuclei in soleus muscle of old rats, indicating that this caspase-independent pathway could play a role in sarcopenia. Very recently, Marzetti et al.[478] analyzed the gastrocnemius muscle of 8-, 18-, 29-, and 37-month old rats for the occurrence of apoptosis. Apoptotic DNA fragmentation, as well as cytosolic and nuclear levels of the caspase-independent apoptotic mediators AIF and EndoG increased progressively with age, paralleling the loss of muscle mass. On the contrary, cytosolic content of cytochrome c remained unchanged over the course of aging and levels of active caspase-9 were slightly elevated in old (29-month) but not in senescent (37-month) rats. Mitochondrial levels of both anti-apoptotic Bcl-2 and pro-apoptotic Bax were significantly increased in the very old (37-month) but not in the old (28-month) animals. The balance between these two mediators, which is believed to regulate mitochondrial membrane stability, was not changed. According to these findings, the authors concluded that mitochondrial caspase-independent apoptotic signaling might play a more prominent role in age-related muscle wasting compared to the caspase-dependent pathway. As a potential apoptosis inducing mechanism they suggested age-associated deletion mutations in the mitochondrial genome.

Oxidative stress has been claimed to lead to the activation of apoptosis via its effect on mitochondria[479]. Age-related increase in ROS may lead to an increase in apoptosis and hence, atrophy and eventually loss of muscle fibers. Another potential mechanism for age-related fiber atrophy or loss is the activation of apoptosis in denervated muscle fibers. Cell death in denervated skeletal muscle is distinct from, but related to classical apoptosis. Hypercondensed chromatin and fragmented nuclei typical of apoptotic cells can be observed in muscle tissue 2, 4 and to a lesser extent 7 months after denervation[469]. Electron microscopy revealed clear morphological manifestations of muscle cell death, with ultrastructural characteristics very similar to those considered as nuclear

and cytoplasmic markers of apoptosis. Another marker of apoptosis, the presence of nuclear DNA fragmentation, was analyzed by the TUNEL (terminal deoxyribonucleotidyl transferase mediated dUTP nick end labeling) method. The number of nuclei with abnormal morphology exceeded by over 30 times the number of TUNEL positive nuclei, indicating that genomic DNA fragmentation is not an obligatory event during atrophy and cell death of muscle cells, or, alternatively, it may occur only for a short time period during this process. In contrast to classical apoptosis, non-inflammatory death of muscle fibers in denervated muscle occurs a long time after removal of myotrophic influence.

Since apoptosis is increased in aged muscles and muscle fibers decrease in size with aging, it was expected that myonuclear number would decrease and a constant myonuclear domain would be maintained. However, studies in humans and rats have indicated that there is no decrease in myonuclear number with age, but rather aged muscles exhibit a smaller myonuclear domain[477,480,481]. Because increased amounts of centrally nucleated fibers have constantly been observed in aged muscles (see above), fusion of satellite cells to replace the nuclei lost by apoptotic nuclear death is a probable scenario[271]. Smaller myonuclear domain in aged subjects may be attributable to impaired function and lower efficiency of aged nuclei, which could be the consequence of oxidative damages.

Together, apoptosis, most likely via the mitochondrial caspase-independent pathway, appears to be elevated in aged skeletal muscle. Both oxidative damages to mitochondria and denervation of muscle fibers are potentially inducing apoptosis. If other signals, such as elevated levels of calcium, could induce apoptosis in age has to be determined. However, it is unclear whether apoptosis contribute to age-associated muscle fiber atrophy or loss. Apoptotic loss of myonuclei in aged muscle is counteracted by fusion of satellite cells. Finally, it is not the loss of myonuclei but rather the decreased myonuclear domain that contributes to fiber atrophy. Alternatively, apoptotic nuclear cell death following denervation or oxidative damage may lead to the death of the entire muscle cell and contribute to the age-related loss of muscle fibers. In such a scenario, a lower myonuclear number would not be found.

1.4.8 The influence of physical activity on sarcopenia

The neuromuscular degeneration observed in aging animals has also been proposed to be due to changes in motoneuronal activity rather than aging per se[423]. Generally, senescence is associated with reduced physical activity in humans[482], although some older people maintain a high level of activity. The tendency to be less active in old age appears to be a universal phenomenon, occurring in different species, such as fruitflies, rodents, monkeys and humans. However, contradictory reports demonstrate the difficulty of the assessment of physical activity. Physical activity, quantitated by three-dimensional accelerometer worn about the waist for one weak, was for example not found to be decreased in old (65-83 years) compared to young (26-44 years) adults[274]. Robbins and Fahim[401] analyzed the locomotor activity in adult (5-9 months) and aged (27-31 months) mice. The daily activity, continuously monitored over 24 hours, was averaged over a 20-day-period and varied greatly between individual mice, with about twofold difference between the least and the most active mice. The mean average daily activity and the mean lowest and highest activity of the two age groups were not significantly different. This result was in contrast with most studies of rats and other mice, in which a decline in locomotor activity of 57-64% was noted between 6 and 30 months[483]. However, previous studies entailed only single or multiple trials over a few minutes duration, which may lead to a less accurate picture of overall activity. Thus, decline in physical activity is not necessarily associated with aging. It may depend on species and environment, and certainly varies greatly between individuals.

Decrease in activity results in general muscle atrophy and a decline in muscle strength. Patrick et al.[151] analyzed body fat and muscle in 73 manual workers at and 1 year after retirement and found an increase in body fat and a decrease in body muscle. The decline in muscle was more pronounced in a subgroup of 22 men who reported that their activity was less after the retirement. Because the assessment of physical activity over a long time period bears a lot of difficulties, bed rest or immobilization is often used as short-term model for decreased physical activity. Data form human studies of experimental bed rest, lower limb unloading, or spaceflight ranging from 2 to 6 weeks reported strength losses in range of 12-25% for knee and ankle extensors[484-492]. Thereby, type specificity was not detected[485,493]. Strength losses have mainly been accounted for by decreased skeletal muscle mass. A 2-3% weekly rate of atrophy has been found in the majority of studies examining healthy individuals after 1-6 weeks of unloading or bed rest[484,485,487,491-497]. However, the decline in strength could not entirely be attributed to the decrease in muscle CSA. Therefore, it has been suggested that the strength loss could also be due to factors resulting in decreased neural input to muscle or decreased specific tension of muscle. Recently, Suetta et al.[498] showed that 2 weeks of immobilization caused decreases in strength, quadriceps volume and specific force in both young and old men. After 4 weeks of retrain initial values of strength and quadriceps volume were reached in both groups, indicating that the changes to immobilization do not include irreversible loss of muscle function or mass. Most likely, fibers were atrophied, but not lost during immobilization.

Data obtained in animal studies are in close agreement with the findings in humans. Reductions in muscle weight and strength were between 20 and 50% after 1-6 weeks of hindlimb immobilization or suspension in adult rats[499-503]. Similar atrophy was observed after unloading in weightlessness during spaceflight in rats[504] and monkeys[505]. In a number of studies a decrease in proportion of type I fibers during unloading has been detected in rats[501,506-510]. However, alterations in fiber type composition were not associated with a change in fiber number[511,512].

Interestingly, an increase in intramuscular connective tissue, regularly observed in sarcopenia, has been found in several studies of hindlimb immobilization in rats[508,513,514] and lower limb suspension in humans[515]. Moreover, a significant linear relationship between physical activity and percent noncontractile content of muscle was found in old, but not in young subjects[274]. Thus, physical activity can modulate the age-related increase in noncontractile muscle components, but appears not to influence the amount of connective tissues in young subjects. Experimentally induced muscle disuse in rats has also been reported to lead to denervation-like alterations[501] and to changes in motor nerve terminals such as increased turnover and sprouting[516]. However, functional parameters, such as miniature endplate potential frequency, specific membrane resistance or resting membrane potential were unaffected by periods of disuse as long as 6 weeks[417,517]. On the other hand, increase in synaptic efficiency (increased fractional release of ACh and faster recovery) and a small spread of extrajunctional ACh sensitivity was observed, indicating that the level of conducted neural activity affected postsynaptic receptor distribution. However, the magnitude of the changes suggest that subtle decreases in normal cage activity with advancing age may not have significant effects on the morphology of the NMJs[518]. The motoneurons seem also not to be affected by decreased physical activity. No changes in morphological or metabolic properties of spinal motoneurons innervating soleus muscle have been observed following 2 weeks of hindlimb unloading in young adult rats, although muscle atrophy was observed[510]. Importantly, all studies concur that immobilization induced changes are to a great extent reversible phenomena, especially if remobilization is intensified by physical training.

Together, decrease in physical activity results in muscle atrophy that shares features with sarcopenia but fails to explain typical characteristics of sarcopenia. Fiber atrophy, loss of strength, increase in intramuscular connective tissue, and remodeling events at the NMJ can be observed in both

immobilization atrophy and sarcopenia. However, loss of muscle fibers has not been observed in models of muscle disuse and increased heterogeneity of fiber thickness, involving atrophy and hypertrophy within the same muscle, is clearly not a result of decreased physical activity. Thus, although a reduction in the amount of physical activity with aging undoubtedly contributes to atrophy, it can't account for sarcopenia. Alteration in physical activity may rather modulate than induce age-related muscular changes. Furthermore, models of immobilization simulate an extreme situation, in which movement is completely abolished. Aging is associated with, at most, a slight decrease in physical activity, and not with complete immobilization. Immobilization may result in effects that are by far more severe than those upon slight reduction of activity. On the other hand, although immobilization does induce a decrease or cessation of muscle activity, the immobilized muscle never become inactivated completely, since the structural continuity of the neuromuscular apparatus is maintained[519]. Similarly, decreased physical activity does not lead to complete blockage of neuromuscular activation. Thus, it does not induce situations where a complete absence of neural activation can occur, which is certainly the case in individual fibers from sarcopenic muscles. Moreover, individuals maintaining a high physical activity also suffer from sarcopenia, indicating that reduced physical activity alone does not account for sarcopenia. Decreased physical activity may rather be a consequence than a cause of age-related neuromuscular deterioration. Sarcopenia, by making physical activity more difficult, may be one of the reasons for being inactive.

Increasing physical activity, on the other hand, can induce muscle growth in young adults[520,521]. However, these adaptations to changes in muscle activation involve modifications in the CSAs of individual muscle fibers rather than in fiber number. There appears to be little, if any, increase in fiber number in response to exercise training in young subjects. Gollnick et al.[522] demonstrated for example that muscular enlargement by synergistic ablation and/or treadmill exercises resulted in increase in muscle weight from 10 to over 100%. Muscle fiber hypertrophy, increase in CSA, but not hyperplasia, increase in fiber number, was detected in soleus, plantaris and EDL muscles of rats. Similarly, overloading of chicken anterior latissimus dorsi muscle, which might be more similar to weight-lifting activities as practiced by man, resulted in enlargement of skeletal muscle that was entirely due to hypertrophy of preexisting fibers and not due to formation of new ones[523]. These findings suggest that exercise training in the elderly probably does also not restore the number of fibers lost to aging. Daw et al.[199] demonstrated that exercise training did not affect the soleus fiber number in aged rats, although muscle mass was 113% of sedentary controls. Although regular exercises can avoid, or at least delay, disability caused by sarcopenia, they cannot prevent a relative decline in muscle mass and performance[174,524-529]. It remains unclear whether the increase in whole muscle size in aged people after resistance training[530-533] results from the hypertrophy of only healthy fibers or whether it also benefits the severely atrophied fibers: the answer to this question will be critical to understand whether exercise training actually affects fundamental aging processes in skeletal muscle or merely slows the consequences of fiber loss and selective atrophy by promoting compensation by healthy fibers.

1.4.9 Biochemical changes in the aging muscle

1.4.9.1 Age-associated changes in protein synthesis

Proteins represent the most important muscle constituents and accounts for about 20% of muscle weight. An imbalance between removal of old and damaged muscle proteins and synthesis of new ones could potentially contribute to the age-associated loss of muscle mass[534]. To understand the biochemical basis for sarcopenia, protein synthesis and breakdown rates have been compared in subjects of different ages. Since muscle contributes less than 30% to the whole body protein

turnover, whole body measurements are inappropriate for determining small alterations in muscle protein synthesis and breakdown. Thus, fractional synthesis rates of mixed or myofibrillar proteins from muscle biopsy samples have been measured. A number of studies demonstrated a progressive decline in fractional muscle protein synthesis with age, indicating that aging is associated with a reduced capacity of skeletal muscle to synthesize new proteins[165,166,535,536]. A decrease of 28% in the synthesis rate of myofibrillar proteins in the elderly has been reported, measured by *in vivo* incorporation of ^{13}C labeled leucine into myofibrillar proteins obtained by muscle biopsy[165]. Whole body protein synthesis, in contrast, was only marginally slower. However, these results have been challenged by other studies[537,538]. Volpi et al.[538] did not find an age-related change in the fractional synthesis or breakdown rate of skeletal muscle protein. The contrast to previous studies was explained by a combination of factors. Any dietary manipulations prior to the study were avoided and only healthy and active people were intentionally selected. Importantly, in contrast to previous studies, not only muscle protein synthesis rate, but also the breakdown and net balance of muscle proteins was measured. Quantification of breakdown is absolutely required to draw any conclusions on the mechanisms leading to the loss of skeletal muscle mass. The authors calculated that, without a change in protein breakdown rate, a 30% reduction in the myofibrillar and mixed muscle protein synthesis rate would have resulted in a net loss of approximately 60% of the muscle mass within one year. This reduction is inconsistent with the natural history of sarcopenia that develops over decades and suggests that the measurements of that large decrease in muscle protein synthesis rate may reflect an acute response to the experimental design. Differences in basal muscle protein turnover between old and young subjects do not appear to explain age-related muscle mass loss.

Importantly, the synthesis rate of mixed muscle or myofibrillar protein represent an average measure of the synthesis rate of several proteins. Different muscle proteins are synthesized at different rates and regulated by different genes. An insight into the pathophysiology of sarcopenia at the molecular level requires measurement of the synthesis rate of smaller groups of proteins or even individual proteins. Rooyackers et al.[142] found for example a substantial age-associated decline in the rate of mitochondrial protein synthesis in skeletal muscle. From young (24 yr) to middle age (54 yr), there was a decrease in mitochondrial protein synthesis rate of on average 40%, whereas no further decrease was observed in individuals at older age (73 yr). In agreement with this finding, a reduction in muscle respiratory capacity with aging has been observed (see above). Balagopal et al.[166] found that myosin heavy-chain synthesis rate declined progressively with advancing age, whereas sarcoplasmic protein synthesis remained constant. A decline in the rate of myosin heavy-chain synthesis could contribute to diminishing contractile function in the elderly. Welle et al.[435] used serial analysis of gene expression (SAGE) to perform a broad search for transcripts expressed at quantitatively different levels in vastus lateralis muscle of young (21-24 yr) and older (66-77 yr) individuals. The mRNAs encoding several mitochondrial proteins involved in electron transport, including several subunits of complexes I and IV, and subunits of ATPsynthase were about 30% less abundant in older muscle, a finding that may be partially explained by the lower mitochondrial volume of aged muscles (see above). Furthermore, the expression of several genes encoding enzymes involved in glucose metabolism was also decreased in older muscle. Beside synthesis, also spatially expression of muscle proteins could be changed with aging. The expression pattern of myosin heavy-chain was altered with aging, resulting in an increased occurrence of single fibers expressing two or more isoforms[180]. Marked alterations occurred in the concentrations of proteins in the sarcoplasmic reticulum. The concentration of the Ca^{2+}-ATPase was reduced in older subjects, whereas the concentration of the Ca^{2+}-channel release protein was actually increased. These age-related alterations in protein expression may contribute to the slowing of muscle contraction with age.

Recently, gene expression in aged skeletal muscle has been analyzed using high-density oligonucleotide microarrays. Differential gene expression pattern indicative of a marked stress response and lower expression of metabolic and biosynthetic genes have been observed with aging in the gastrocnemius muscle of mice[539-541]. Mediators of stress responses, such as oxidative damage inducible and DNA damage inducible genes, increased in expression with age, whereas the expression of genes associated with mitochondrial function and turnover was decreased. Furthermore, aging was characterized by inductions of genes involved in neuronal growth. These findings imply accumulation of oxidative damage, reduction in energy metabolism and nerve sprouting in aged skeletal muscles. A similar transcriptional profiling on the soleus muscle of young compared to old rats revealed an extensive downregulation of extracellular matrix genes[542]. 24% of the downregulated genes have a biological role in the extracellular matrix and cell adhesion. The genes with increased expression with age were involved in immune response, proteolysis, ubiquitin-dependent degradation and stress response. Interestingly, the expression of the mRNA of several subunits of AChR was increased in the old compared to young soleus muscle, probably reflecting the appearance of extrajunctional receptors and denervated muscle fibers in aged muscles. Kayo et al.[543] examined mRNA from vastus lateralis muscles of young adult, middle-aged and old rhesus monkeys. Aging was associated with reduced expression of genes involved in mitochondrial electron transport and oxidative phosphorylation, and with upregulation of transcripts involved in oxidative stress responses, reinnervation of muscle fibers, and inflammatory or immune responses. Later, the gene expression profile of aging in human vastus lateralis muscle was established[544]. In accordance with previous findings, the expression of genes with a function in energy metabolism and mitochondrial protein synthesis was decreased in older muscle, whereas the expression of components of the ubiquitin-proteasome proteolytic pathway was increased. Expression of numerous genes involved in stress responses, hormone/cytokine/growth factor signaling, control of cell cycle and apoptosis, and transcriptional regulation appeared to be affected by aging. However, these changes tended to be of low magnitude and the analysis is complicated by the heterogeneity of human muscle samples. Therefore, the so-called molecular signature, the smallest number of gene expressions that is required to identify aged muscle, was established[545]. The molecular signature of sarcopenia among 10 young and 12 older male subjects included 45 genes. Interestingly, genes involved in mediating cellular response to inflammation and apoptosis were expressed at higher levels in aged skeletal muscle, suggesting an involvement of these processes in sarcopenia. More information about genes that show age-related differences in expression are important for the understanding of the molecular events underlying sarcopenia and may lead to novel treatment strategies.

1.4.9.2 Influence of age-associated endocrine changes on sarcopenia

The growth and maintenance of muscles is regulated by a number of hormones and growth factors. Some of these are produced in remote organs and are transported to muscle in the blood (endocrine signals), while others are produced locally in the muscle (autocrine and paracrine signals). There is an age-associated decline of the circulating levels of several hormones, such as testosterone, estradiol, growth hormone (GH), and insulin-like growth factor-1 (IGF-1). Serum Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and cortisol levels, in contrast, are increased in old age[529]. All these age-associated hormonal changes might potentially contribute to sarcopenia.

1.4.9.2.1 Steroids

Androgens, such as testosterone, regulate muscle mass in humans[546]. Testosterone administration has been shown to increase muscle size and strength, and fat-free mass in normal men in a dose

dependent manner[547]. Testosterone binds to androgen receptors, which are predominantly expressed in satellite cells and myonuclei[548,549]. It increases satellite cell number by potentially regulating their proliferation and differentiation[549,550], and leads to greater numbers of myonuclei in larger myofibers[551]. There is increasing evidence that androgens interact with IGF-1 in skeletal muscle[552]. In rat diaphragm muscle, a dose dependent increase in IGF-1 mRNA expression occurred following exposure to androgen[553]. When harvested bovine male satellite cells were treated with various androgen concentrations, a dose dependent increase in IGF-1 mRNA expression occurred[554]. Similarly, increase in serum IGF-1 levels has been positively correlated with concentrations of testosterone administrated to young men[547].
Between the ages of 25 and 86 years, testosterone levels decrease by 60% and 28% in healthy males and females, respectively[259-261]. In about 60% of men over the age of 65, testosterone levels decrease to below the normal values in young men[163]. Due to the anabolic effects of testosterone on skeletal muscle, it has been proposed that its decline could result in a loss of muscle mass[555]. Indeed, a relationship between serum testosterone levels and muscle strength in older man has been demonstrated by several studies. In contrast, a decrease in testosterone has not been linked to a decline in muscle mass or strength in women[132,556,557]. Several studies have examined the effect of testosterone replacement therapies and found a significant increase in muscle mass and strength, fat-free mass, protein synthesis and bone density in elderly men following testosterone application[558-560]. Like in young men, increase in muscle mass and strength and fat-free mass has been shown to correlate with the amount of testosterone administrated[561]. Older men are as responsive as young men to the anabolic effects of graded doses of testosterone on skeletal muscle. However, doses greater than 100 mg applied by weekly injections are needed to induce significant anabolic effects. Low doses of testosterone appear not to increase muscle mass and strength in the elderly[562]. Furthermore, increase in muscle mass may not always result in improved strength[563]. Together, decreasing testosterone level may not play a prominent role in the etiology of sarcopenia, since it fails to explain most of the sarcopenic phenotype. Moreover, a correlation of decreasing testosterone concentrations and muscle mass in aging women is missing, despite women also suffer from sarcopenia. It's not very likely that fundamental sarcopenia promoting processes are different between men and women. Decreasing estradiol levels in women do also not mimick the effects of decreasing testosterone levels. Estradiol levels abruptly decrease during menopause. The decline in muscle mass, in contrast, progresses gradually over decades, suggesting only marginal, if any, role of decreased estradiol levels on sarcopenia.

1.4.9.2.2 The growth hormone/insulin-like growth factor-1 axis

Many of the responses regulated by GH are primarily mediated by IGF-1 in tissues throughout the body[564]. IGF-1 is produced by the liver, but also by the skeletal muscle and is important for the pre- and postnatal development of skeletal muscle. There are local as well as systemic forms of IGF-1 that have different functions. The IGF-1 gene is subject to alternative splicing, resulting in at least two variants that are expressed from skeletal muscle including the so-called mechano growth factor (MGF)[565].
It is generally accepted that serum IGF-1 concentrations decrease gradually with age[163,566,567]. However, it was for a longtime unknown whether skeletal muscle IGF-1 expression change during aging. Hamilton et al.[568], using a ribonuclease protection assay, found that skeletal muscle IGF-1 mRNA levels changed during maturation (decrease of 39-49% between 3 and 12 month old rats) but not during aging (12-24 month old rats) in the gastrocnemius muscles of rats. Recently, using quantitative RT-PCR on human muscle tissue, it has been demonstrated that older men tend to have lower IGF-1 mRNA levels in muscle[569]. In older muscle (62-77 yr) IGF-1 mRNA concentration was about 25% less, per ng total RNA, than in young adult muscle (21-31 yr). One

third of the older men had IGF-1 mRNA levels below the lowest concentration detected in young men. Thus, IGF-1, as an important regulator of muscle mass, could influence the development of sarcopenia.

IGF-1 production is sensitive to increases in loading state of the muscles. Overloading of skeletal muscle produces hypertrophy and is associated with increase in IGF-1 mRNA and peptide level[570], whereas unloading-induced atrophy is associated with decreased IGF-1 mRNA expression[571]. Especially one of the skeletal muscle isoforms of IGF-1, MGF, is expressed in response to physical activity and is responsible for the hypertrophy of a muscle in response to physical exercise. Consequentially, injection of MGF cDNA into skeletal muscle of mice results in a 25% increase in the mean muscle size within 3 weeks with a similar increase in muscle strength[572]. Similarly, Barton-davis et al.[573] demonstrated that virus-mediated overexpression of IGF-1 in differentiated skeletal muscle fibers promoted an increase of 15% in muscle mass and 14% in muscle strength in young adult mice. Interestingly the ability to increase IGF-1 expression in response to mechanical overload is reduced in older muscles of rats[160] and humans[574]. The increase in IGF-1 expression following muscle overloading is an adaption mechanism that is impaired in senescence.

Age-dependent decrease in IGF-1 concentrations, together with the ability of IGF-1 to induce hypertrophy in young muscles, suggests that low level of IGF-1 or unsuccessful increases of IGF-1 level after induction may contribute to the etiology of sarcopenia. Several studies assessed the influence of sustained or even increased IGF-1 expression or application in old muscles. In old mice, viral mediated IGF-1 overexpression in skeletal muscle prevented age-related loss of skeletal muscle function, resulting in a 27% increase in strength compared with untreated muscle[573]. Muscle mass and fiber type distribution were maintained at levels similar to those in young adults. The authors proposed that these effects are primarily due to stimulation of muscle regeneration via the activation of satellite cells by IGF-1. There is evidence that IGF-1 is involved in the replication of satellite cells which is important for the continuing maintenance of muscle mass and function. The application of IGF-1 onto immobilized old skeletal muscle restores the proliferative potential of satellite cells and partially rescues the lost muscle mass[284]. Localized IGF-1 transgene expression in skeletal muscle of the mouse preserved muscle architecture and regenerative capacity in aged muscles[575] and partially rescued age-dependent alterations in neuromuscular innervation[576]. Age-related alterations of nerve terminal branching, of number, length, area and density of postsynaptic folds, and of postsynaptic α-bungarotoxin stained area were partially or completely rescued by sustained overexpression of IGF-1 in skeletal muscle. IGF-1 appears to enhance the reinnervation of muscle fibers and could be relevant to the repair and preservation of muscle function in more than one way. Evidence for different roles of IGF-1 in preservation of muscle function also comes from denervation studies in transgenic mice, which overexpresses IGF-1 specifically in differentiated myofibers[317]. The rate of denervation-induced atrophy was reduced by approximately 30% in these mice and myofibers with larger CSA were preserved. Here, innervation was not involved and the beneficial influence of IGF-1 was attributed to reduced protein breakdown. IGF-1 is also involved in age-related ECU by regulating transcription of DHPR[224,577], which plays a key role in intracellular Ca^{2+} handling and muscle contractility (see above).

The cellular actions of IGF-1 are modulated by the actions of a family of six IGF binding proteins (IGFBP1-6). Although the precise role of these IGFBPs is poorly understood, the disruption of IGFBP expression has been found to impair myoblast proliferation, differentiation and maturation[578-581]. The circulating level of the IGFBP undergoes age-related alterations, thereby influencing the availability or the action of IGF-1. What effect this may have on skeletal muscle in detail has yet to be fully elucidated[582].

The level circulating GH is also subject to age-related alterations. It is reduced to about one-third in aged (older than 70 years) compared to young proband (younger than 20 years)[583]. GH is known to

induce liver IGF-1 expression, but it was longtime unknown if circulating GH influences skeletal muscle IGF-1 gene expression[584,585]. Hameed et al.[586] showed that GH treatment increased IGF-1 levels in elderly man and when combined with resistance exercise more is spliced towards MGF, resulting in 450 % increase in MGF mRNA levels. This was significantly more than the change induced by resistance training alone (150%). However, treatments with GH, even in combination with resistance training, didn't result in a significant increase in muscle CSA, compared to resistance training alone[587]. Although the senescent muscle remains responsive to hypertrophy, there is a limit to the improvement that can be obtained given the loss of muscle fibers that has occurred.

In summary, IGF-1 can beneficially influence aging muscles and delay sarcopenia. This has been attributed to increased regeneration, preservation of innervation, increased reinnervation, and reduced muscle protein breakdown. However, it's unclear whether sustained IGF-1 levels can counteract the fundamental sarcopenia promoting processes and, the other way round, whether decreasing IGF-1 levels actually cause sarcopenia. Increased IGF-1 level may rather induce regeneration or reinnervation than prevent the pathogenic process leading to sarcopenia per se.

1.4.9.2.3 Inflammation and sarcopenia

Irreparably damaged skeletal muscle undergoes autolysis, followed by an inflammatory response that is critical for efficient muscle regeneration. A number of studies have suggested a link between aberrant inflammatory/cytokine signaling and sarcopenia[588-591]. Especially TNF-α and IL-6 have been implicated in the inflammatory response in skeletal muscle. An age-associated increase in the expression of these inflammatory markers is thought to contribute to the etiology of sacopenia[592,593].

TNF-α is a central mediator orchestrating cellular inflammatory and apoptotic signaling pathways. It is primarily produced by activated macrophages and has been evidenced in patients with heat failure, sepsis, and other inflammatory diseases that result in secondary muscle weakness. Elevated levels of TNF-α in aged muscles have been postulated to increase apoptosis and to impair the inflammatory response to injury[594]. Phillips and Leeuwnburgh[595] investigated the effects of age on TNF-α levels and on TNF-α signaling cascade in slow soleus and fast superficial vastus lateralis (SVL) of adult (6 month old) and aged (26 month old) rats. In the aged animals, loss of muscle fibers was accompanied by an increase in plasma levels and myocyte expression of TNF-α. Signaling through TNF-α was distinct in aged soleus and SVL. In soleus muscle, a greater capacity to cultivate inflammatory signaling through NF-κB was observed, whereas in SVL TNF-α stimulated apoptotic signaling was elevated. Consistent with this finding, reduction in muscle mass, CSA, and fiber number were greater in SVL than in soleus muscle. The decision of selecting life or death signaling pathways in response to TNF-α may be distinct according to fiber type and appears to be linked to the extent of fiber loss experienced in the aging muscle. Thus, TNF-α transmitted signals may constitute a proponent in the pathogenesis of sarcopenia.

Il-6 plays a central role in host defenses and acts upon a wide variety of tissues. It influences such diverse processes as immunoglobulin secretion in B-cells, maturation of megakaryocytes and neuronal cells, and differentiation of cytotoxic T-cells. Serum IL-6 levels are increased with age and correlate with the functional status among subjects over 70 years old[596]. Elevated levels of IL-6 can initiate muscle wasting[597] and experiments using IL6-deficient mice showed that IL-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy[598]. Functional overloading did not lead to muscle hypertrophy in IL-6 deficient mice due to reduced proliferation of satellite cell and subsequent impaired accretion of myonuclei to muscle fibers.

Direct evidence for TNF-α and IL-6 to play a causal role in sarcopenia is missing despite the age-related increase in their expression and despite their involvement in muscle mass regulatory

processes. Other cytokines whose activities are closely related to TNF-α, or IL-6 such as IL-1 and interferon-gamma also cause cachexia and may potentially influence sarcopenia[599]. Aging appears to be accompanied by constitutive inflammatory processes that are reflected by elevated cytokine levels. However, whether inflammation causes sarcopenia or, alternatively, sarcopenia leads to inflammation is not yet clear.

1.4.9.2.4 Myostatin

Myostatin (growth/differentiation factor-8, GDF-8), a member of the transforming growth factor-β (TGF-β) superfamily, negatively regulates pre- and postnatal myogenesis. It inhibits satellite cell activation and muscle regeneration postnatally. Myostatin was first discovered by McPherron et al.[600] who demonstrated that a phenotype of exaggerated muscle hypertrophy correlated with mutations in the myostatin gene. Such knockout mutations of myostatin in animals caused the so-called double-muscled phenotype. Double-muscled animals are characterized by an increase in muscle mass of about 20%, due to general skeletal muscle hyperplasia, an increase in the number of muscle fiber rather than in their individual diameter[601]. Research on aged myostatin mutant mice indicates that the absence of myostatin reduces age-related sarcopenia[602]. Whereas aged wild-type muscle became increasingly oxidative and fiber atrophy was prominent, fiber type switching was not observed and atrophy was minimal in aged myostatin-null mice. In addition, muscle regeneration following notexin injury was accelerated in myostatin-null muscle. Beside positive acting factors, negative regulators of myogenesis may also play a role in aging of the skeletal muscle. However, a direct involvement of myostatin in sarcopenia has not been observed and, importantly, myostatin levels do not change according to age. Myostatin mRNA concentrations, assayed using quantitative RT-PCR, were similar in young and old human muscle[569].

1.4.9.2.5 Myogenic regulatory factors (MRF)

The family of myogenic regulatory factors (MRFs), including MyoD, myogenin, Myf-5, and MRF4, are involved in regulation of myogenic cell differentiation during embryogenesis. MRFs are characterized by basic helix-loop-helix sequences that bind DNA and transactivate most muscle-specific genes by recognizing a consensus sequence present in their regulatory region. MyoD has been especially related to proliferation of satellite cells, whereas myogenin has been related to muscle differentiation and expression of muscle-specific proteins. These MRFs are generally thought to be marker of skeletal muscle growth and hypertrophy, since they can modulate satellite cell division and their incorporation in mature muscle fibers. MyoD and myogenin transcripts are expressed at high levels in muscles of newborn mice and their level continuously declines through postnatal life to become virtually undetectable in the adult mouse[603]. In the muscles of older mice, these transcripts are again expressed at high levels. MRF4 transcripts, in contrast, are present at a constant level throughout an animal's life. Conversely, the expression of Myf-5 conspicuously increases in adult and senile muscle. Complex regulatory networks are activated during muscle aging with each of the MRFs showing a distinct pattern of expression. Increased expression of MRFs has not only been demonstrated in muscle of aged mice, but also in regenerating muscle of young rodents[604-606], and in denervated muscles[607-609], outlining the role of MRFs in muscle regeneration and innervation. Marsh et al.[610] assessed MRF mRNAs during regeneration of skeletal muscle after bupivacaine injection in young, adult, and old rats. Without treatment, Myogenin, MyoD, and MRF4 mRNA all were increased in old compared to young and adult muscles, a finding that may reflect a continued attempt to generate new muscle fibers and ameliorate muscle atrophy in old muscle. During regeneration at 5-14 days after bupivacaine injection, expression of the MRF genes was increased in young, adult and old rats. Thereafter,

MRF mRNA levels returned to control values in young and adult rats. In old rats, however, only MRF4 expression returned to control level, whereas myogenin and MyoD expression remained upregulated. The authors proposed that either the ability to downregulate myogenin and MyoD mRNAs in regenerating muscle is diminished in old muscles, or the continuing myogenic effort includes elevated expression of these mRNAs. Since myogenin and MyoD levels are sensitive to the innervation status of the muscle, it is possible that their prolonged expression during regeneration in old muscles could be related to incomplete reinnervation. Impaired reinnervation of denervated or newly synthesized muscle fibers is thought to substantially contribute to sarcopenia (see above). MRFs have also been suggested to play a role in the control of skeletal muscle fiber type composition. Voytik et al.[611] found that different muscles accumulated different proportions and combinations of MRF transcripts. Myogenin mRNA was for example detected at higher levels in muscles exhibiting a slow-twitch phenotype, whereas MyoD transcripts were predominantly found in fast-twitch muscles. Therefore, the authors proposed that MRFs could be involved in both the initial establishment as well as maintenance of fiber type diversity in the developing organism. Consequentially, age-related changes in MRF expression pattern could contribute to the shift in fiber type frequencies seen in sarcopenia.
Together, the expression of MRFs is subject to age-related changes that are amplified in senescence. However, these changes might rather be a consequence than a cause of sarcopenia. The elevated demand for regeneration in aged muscles in concert with incomplete innervation induces MRFs to upregulate the expression of muscle specific genes needed for regeneration and innervation. Such genes include AChR and other components of the NMJ as well as genes involved in satellite cell activation, differentiation and fusion. Thus, increase in MRFs is counteracting and not inducing sarcopenia. Sustained high expression of MRFs in age indicates that the muscle regeneration system is still working although, finally, not successful. Unsuccessful regeneration and incomplete innervation is not likely to be caused by impaired MRF regulation or action, since upregulation of MRFs and of muscle specific genes controlled by them can be detected.

1.4.9.2.6 Systemic alterations do not cause sarcopenia

Age-related endocrine changes may not play a major role in the etiology of sarcopenia, since they fail to explain important characteristics of the sarcopenic phenotype. It is for example unclear how systematic alterations, such as reduced blood concentrations of anabolic hormones and growth factors, increased levels of cytokines, and decreased protein synthesis could explain the selective pattern of atrophy that results in the heterogeneous distribution of fiber thickness with aging[423]. The severe atrophy of some fibers accompanied by compensatory hypertrophy of other fibers within the same muscle can't be caused by a process that affects the whole muscle. Clearly, the etiology of sarcopenia must involve processes intrinsic to individual myocytes, or the motoneurons that innervate them, in such a way that only some cells are affected whereas others are not. Systematic alterations could intensify the problem without being the cause.

1.4.10 Treatment or prevention of sarcopenia

Age-related endocrine changes, nutritional deficiencies and physical inactivity in the elderly are thought to contribute to the pathogenic process leading to sarcopenia. Therefore, the substitution of anabolic hormones and growth factors, such as testosterone, growth hormones, and IGF-1, as well as agents that reduce the levels of inflammatory cytokines have been under investigations for the development of therapies against sarcopenia. As alternative or complementary approaches, programs aimed at changing nutritional behaviors and increasing physical activity, have been composed.

Exercise training in the elderly is clearly beneficial in restoring strength and function[532]. Coggan et al.[530] assessed skeletal muscle adaptations to 9-12 months endurance training in 60- to 70-yr-old mean and women. They found a 23% increase in maximal O_2 consumption, an increase in the area of both type I (12%) and type II (10%) fibers, increased capillary density, and increased activities of mitochondrial enzymes. These adaptations of the skeletal muscles of older men and women are very similar to those observed in young people. Another study suggests that endurance training attenuates the age-associated loss of relative strength (strength per muscle area or volume) but fails to prevent the age-associated loss of muscle mass or absolute strength[174]. Physical exercises may well be capable of increasing muscle strength[612], but longtime prevention of age-related decline in strength may not be possible. Similarly, endurance training was associated with 60-100% higher muscle citrate synthase activity and improved metabolic responses in young and older men but could not prevent an age-related decrement in these variables or an age-related decrease in muscle mass[524]. Relatively short time of resistance exercises also suppresses or attenuates the age-related increase in skeletal muscle TNF-α levels and the reduction in protein synthesis rate in concert with a substantial increase in muscle strength[612]. Together, exercise training shows clearly beneficial effects but it is questionable whether it fundamentally reverses sarcopenia. Several lines of evidences rather indicate that physical exercises only attenuate or delay the development of sarcopenia but fail to stop or even reverse it.

Nutritional or hormonal interventions in treating the consequences of sarcopenia or slowing its progress can also be partially successful. Anabolic hormone replacement, especially in combinations with aerobic, resistance, and stretching exercise programs can have beneficial effects on sarcopenia in the elderly[162]. Supraphysiological testosterone doses induce substantial gains in muscle mass and strength in older men, but these high doses are associated with a high frequency of adverse effects[561]. Such adverse effects of testosterone therapy include increased hematocrit, increased size of the prostate, increased prostate specific antigen level, and unfavorable effects on lipid profiles[613]. Testosterone is currently not recommended for the treatment of sarcopenia and a careful evaluation of potential risk and benefits has to be performed.

The effect of growth hormone replacement in the elderly population remains controversial. A number of studies have shown that supplementation with GH to stimulate IGF-1 levels to levels observed in young adults can increase lean body mass, decrease adipose tissue, and increase thigh strength[614,615]. However, a number of studies that combined GH administration with resistance training in elderly men failed to show beneficial effect of GH above exercise alone, in terms of muscle strength or muscle protein synthesis[616,617]. Moreover, significant adverse effects due to GH administration, including fluid retention, carpal tunnel compression, gynecomastia, and arthralgias have been reported[618,619]. Together, exclusive pharmalogical interventions, such as growth hormones and testosterone, increase lean mass (and muscle mass), but do not alter strength much and bears substantial risks of adverse effects[620].

Blackman et al.[621] assessed the effects of GH and sex steroid applications on lean body mass, fat mass, muscle strength and maximum oxygen uptake in 57 women and 74 men at ages between 65 and 88 years. GH applications with or without sex steroids resulted in increased lean body mass and reduced fat mass in both aged women and men. Only sex steroids together with GH increased muscle strength marginally and maximum oxygen uptake in men, whereas in women no treatment had an effect on strength or cardiovascular endurance. Adverse effects, however, were frequent and the authors concluded that GH interventions in the elderly should be confined to controlled studies. Recently, in a placebo-controlled randomized trial of GH, testosterone or both, significant effect on muscle size and body composition was only found in the combination group[562].

Caloric restriction (CR) without malnutrition could be a saver strategy to prevent or delay sarcopenia[622]. Restricting energy intake to 60% of *ad libitum* evokes an extension of maximal

lifespan by approximately 25-40% in rodents and better preserves organ function[623]. The maximal life extension effect in humans may not be as pronounced, but the health benefits of CR in humans are considerable[624,625]. The effects of CR on skeletal muscle and sarcopenia have been demonstrated in several studies. Mild CR (8%) prevented age-associated reduction of plantaris fiber CSA and increase in extramyocyte space in old rats[626]. Moderate CR (30-40%) delayed age-related loss of muscle mass in rhesus monkeys[627] and attenuated age-associated reduction of muscle CSA and fiber number in rats[595]. However, beneficial effects of CR on muscle mass and fiber number was not always found. Food restriction to 65-70% of normal levels did for example not prevent age-related loss of fibers or mass of rat soleus and EDL muscles[199]. Many of the different molecular pathways that are affected by CR, involve alterations of mitochondrial function. The age-related decline in activities of ETC complexes observed in mice with *ad libitum* food access was completely prevented by CR[437]. CR also reduces the prevalence of mtDNA deletion mutations and the abundance of fiber displaying ETS abnormalities in old rodents. The preservation of mitochondrial structural and functional integrity by CR is believed to result from the reduction of oxidative stress and damage. Reduction in mitochondrial ROS generation and increase in mitochondrial protein turnover appear to be the important aspects of the effects of CR on mitochondria. Consequently, CR prevented the 50% decline in muscle mass-specific maximal aerobic function seen between young-adult and aged rats[429]. Reduction in oxidative stress may also promote the ability of CR to attenuate age-associated signs of apoptosis in rat gastrocnemius muscle, possibly by preventing age-associated increase in serum levels and myocyte expression of TNF-α[476,595]. Finally, CR prevented most of the age-associated alterations in skeletal muscle gene expression, analyzed by oligonucleotide microarrays[539-541]. CR resulted in a metabolic reprogramming characterized by a transcriptional shift toward energy metabolism, increased biosynthesis, and protein turnover and reduced the expression of genes associated with stress response and DNA repair. Together, CR may be a safe and efficient strategy to delay the onset of sarcopenia. However, beneficial effects on muscle strength and function have not yet been demonstrated and are essential for a strategy to counteract sarcopenia. Moreover, most of the successful studies on the beneficial effects of CR have been preformed with rodents and to a lesser extend monkeys. Human studies are crucially needed to confirm the beneficial influences of CR on sarcopenia and generally expectation of life.
All therapies applied so far could only partially counteract the loss of muscle strength, without principally reverse the deleterious effects of sarcopenia. Without knowledge of the basic mechanisms underlying sarcopenia, it will not be possible to efficiently treat sarcopenia.

1.4.11 Sarcopenia in *C.elegans*

The nonparasitic nematode *Caenorhabditis elegans* is a microscopic organism that has been found during the last decade to be a powerful genetic system in which to study molecular and biochemical events in organismal aging[628]. Under laboratory conditions, worms develop from an embryo to sexual maturity in 3 days, and then reproduce over a 3- to 4-day period. The usual total adult lifespan is 12 to 18 days. Recently, it has been found that aging worms developed sarcopenia starting at an age of about 7 days[629]. In *C. elegans* sarcopenia was characterized by reduction in muscle cell size due to loss of cytoplasm and myofibrills, and by disorganization among the remaining myofibrils. The nervous system of the worms, on the other hand, remained largely intact during aging. Even severely compromised animals showed only little if any neuronal cell death and no major loss or restructuring of neuritis or nuclei, indicating that at the cellular level the nervous system was well preserved in aging *C. elegans*. However, changes in biophysical properties, such as nerve conduction and synaptic transmission, could not be ruled out. Affected

worms showed a progressive decline in mobility and poorly coordinated movements evoked only by direct stimulation.
Interestingly, like in mice and men, sarcopenia did not develop in a stereotyped fashion at a given age. Instead it developed at different ages and at different rates in a cohort of genetically identical worms grown in culture together. This is surprising for an organism in which genetic or developmental variation between animals do virtually not exist. Stochastic factors appear to significantly contribute to senescent decline. Keeping this finding in mind, considerable variation of the onset and developmental rates of sarcopenia between individuals in inbred rodent strains or in humans, where genetic or developmental variations are huge compared to the worms, becomes equitable. Moreover, sarcopenia served as the best single predictor of mortality in an aging population of worms. It was a better predictor of mortality than chronological age, which suggested that sarcopenia represented the true biological age of a given worm. Consistent with this association, worms carrying the *age-1* mutation, which extended the lifespan by 60 to 100%, showed corresponding delays in the appearance of sarcopenia. Sarcopenia could be a measure of biological age whose development is directly associated with the basic cellular and molecular mechanisms involved in aging[628].
The finding of sarcopenia in worms questions several influences that have been claimed to contribute to sarcopenia in humans. The hormonal milieu of the worm is for example different from those of mammals and many hormones linked to sarcopenia, such as IL-6 and sex hormones, are lacking. Also, in contrast to mammals, in which low levels of IGF-1 have been associated with sarcopenia, in worms, reductions in IGF-1 signaling prevent sarcopenia and result in increase in longevity. Thus, age-related changes in hormone levels may not induce sarcopenia in worms and, by inference, mammals. Furthermore, during aging, the worm nervous system remains largely intact, excluding the death of motoneurons as pathogenic mechanism inducing sarcopenia. However, it might be rather alterations in synaptic structure and function than extensive neuronal loss contributing to age-related cognitive and motor deficits in higher organisms. Thus, higher resolution of synaptic function and NMJ structure in nematodes will be required to identify molecular and subcellular changes that accompany neuronal aging in *C.elegans*. Alternatively, there is some evidence that the causes of sarcopenia might rather lie within the aging muscle cell itself. Oxidative damage and mitochondrial dysfunction are for example candidate mechanisms. Interventions aimed at preventing oxidative damage and protein misfolding extended the worms lifespan, although the effects on sarcopenia have not been examined[630-632].

1.4.13 Conclusion

A critical reduction in muscle mass, strength, and number of muscle fibers, increased heterogeneity of fiber size, fiber type grouping, and signs of muscle regeneration are the most prominent pathological hallmarks of sarcopenia, the form of skeletal muscle atrophy that occurs at advanced age. Available evidence suggests that muscle fiber reduction may be due to denervation and incomplete reinnervation of neuromuscular connections that occurs lifelong but accelerates with aging. A strong regenerative phenotype of senescent skeletal muscles indicates the activation of repair mechanisms that involve activation, proliferation and differentiation of satellite cells to induce hypertrophy and to form new myotubes. Whereas compensatory hypertrophy can partially counteract muscle atrophy, the loss of muscle fibers can't be prevented. Final maturation and incorporation of regenerated muscle fibers appears to be impaired, most likely due to the absence of correct innervation. Sarcopenia coincides with the loss of a substantial fraction of the motoneurons and severe deterioration of the NMJs. These events are candidates that may prevent effective regeneration and could substantially contribute to the pathogenic ensemble leading to sarcopenia.

An age-related decrease in IGF-1, which is known to preserve neuromuscular architecture and facilitate innervation in senescence, may contribute to the impaired regeneration phenotype. Another interesting mechanism for a fundamental sarcopenia promoting role is the damage to skeletal muscle mitochondria by reactive oxygen species that accumulates over a lifetime and results in the removal of the muscle cell by apoptosis. Followed by impaired regeneration due to incorrect innervation, these events may form the core mechanisms underlying sarcopenia. However, numerous other age-related changes that may contribute to the etiology of sarcopenia have been described. Most of them can explain certain features of sarcopenia, but not the whole phenotype and may rather contribute to than actually cause sarcopenia. Other age-related changes, including decreased physical activity as well as nutritional, metabolic and hormonal alterations, may rather be a consequence than a cause of sarcopenia. The understanding of the influence of the different sarcopenia promoting processes may help to develop better therapies to counteract the age-associated loss of muscle mass and function.

1.5 Aim of this study

Recent studies showed that the NMJ requires the protective action of the proteoglycan agrin, in order to escape from a dispersal mechanism controlled by synaptic activity[21,32]. We recently found that agrin is cleaved by the neuronal serine protease neurotrypsin at two homologous, highly conserved sites, resulting in the dissociation of the C-terminal domain containing the NMJ-organizing and protecting function[119]. Excessive cleavage of agrin at the NMJ following neurotrypsin overexpression in motoneurons resulted within days in severe NMJ alterations, very similar to the phenotype of agrin-deficient mice[13,26,31]. The aim of this study was to investigate the influence of neurotrypsin overexpression and, hence, excessive agrin cleavage, on the NMJs and skeletal muscles of adult mice. The neurotrypsin-overexpressing mice are viable but show clear motor deficits, including muscle weakness and impaired motor coordination. To elucidate the role of proteolytic agrin cleavage at the NMJ on skeletal muscle structure and function, skeletal muscles and their NMJs have to be investigated in conditions with enhanced agrin cleavage. The aim is to learn more about the function of agrin and its cleavage for the maintenance of the NMJ in adult mice. Moreover, we are interested in the interplay between nerve and muscle, especially in a situation where it is disrupted. The reaction of the neuromuscular system to disturbances could give insights into the mechanism of neuromuscular degeneration, regeneration and repair. The increase of our knowledge of such processes is of high importance in regard to numerous neuromuscular diseases, whose underlying mechanisms are still poorly understood.

Furthermore, we would like to address the question whether the pathogenic processes ending with sarcopenia may result from impaired NMJs. Therefore, histopathological alterations in neurotrypsin transgenic mice have to be compared with those of sarcopenia. As in conditions with enhanced agrin cleavage, deterioration of the NMJ has been observed with aging[92-95,148,399-401], and could result in partial or total loss of the postsynaptic specialization, in muscle fiber denervation, and, finally, in atrophy and loss of muscle fibers. NMJ destabilization could be the onset of a final common pathway resulting in sarcopenia.

2 METHODS

2.1 Animals

Neurotrypsin-deficient mice

The generation of neurotrypsin-deficient mice (Ntd) has been previously described[119,121]. The last two exons of the neurotrypsin gene, coding for the protease domain, have been deleted in these mice. Neurotrypsin-deficient mice do not express a functional form of neurotrypsin, which results in the absence of the neurotrypsin-specific cleavage fragments of agrin, agrin-90 and agrin-22.

Transgenic mice for conditional overexpression of neurotrypsin

The generation of neurotrypsin transgenic mice has been previously described (Bolliger et al., in preparation). Neurotrypsin and agrin were overexpressed under the control of the promoter of the Thy1.2 allele of the Thy1 gene[633]. The Thy1.2 expression cassette was reported to drive transgene expression pan-neuronally, with a relatively late onset around postnatal days 6-10[634]. Therefore, expression of neurotrypsin and agrin under the control of the Thy1 promoter should ensure that earlier developmental stages are not perturbed by the presence of excessive amounts of transgenic neurotrypsin and agrin. However, all transgenic animals died within 2-3 weeks after birth. The result suggested an earlier activity of the Thy1 promoter than expected. To circumvent early lethality, we generated transgenic mice bearing a dormant (conditional) transgene by insertion of the transcriptional stop signal flanked by loxP sites between the Thy1 promoter and the neurotrypsin-encoding segment. To generate the pThy1-loxP-Stop-loxP-neurotrypsin construct the loxP-flanked transcription stop sequence from the pBS302 plasmid (Invitrogen) was inserted in either direction between the Thy1 promoter and the neurotrypsin-coding segment. The loxP-Stop-loxP cassette completely abolished the lethal phenotype that was observed with the constitutively expressed transgene not only when it was inserted as initially described[635,636], but also in the inverted orientation. The transgenic lines selected for this study contained the inverted orientation of the loxP-Stop-loxP cassette. They were backcrossed for ten generations to C57BL/6J to produce the congenic strains, B6;C3-*Tg*(PRSS12) 491, 493, 494 Zbz and B6;D2-*Tg*(Prss12) 497, 533 Zbz.

Selective overexpression of neurotrypsin in spinal cord motoneurons

Mice bearing the conditional neurotrypsin transgene were crossed with mice expressing Cre recombinase under the control of the motoneuron-specific Hb9 promoter [B6.Cg-*Tg*(Hb9-Cre) mice[14]; provided by S. Arber, University of Basel, Basel, Switzerland] to induce recombination and selective activation of the Thy1-neurotrypsin transgene in motoneurons. This procedure allows maintenance breeding of the transgenic mouse lines bearing the transgene in a dormant state and to generate offspring overexpressing neurotrypsin on experimental demand. Moreover, the selective activation of the transgene in motoneurons was possible. We found that all transgenic animals generated by Cre recombinase-mediated excision of the loxP-Stop-loxP cassette were viable and did not exhibit any gross abnormalities. For the present study 493xHb9-Cre, 497xHb9-Cre, and 533xHb9-Cre were used. For convenience and to underlie the motoneuron-specific expression pattern these mice are referred to as Nto-moto and Nto-moto2. The expression of the transgene was verified at the protein level by Western blotting of neurotrypsin (**Fig. 12b**, **19b**). The proteolytic activity of the transgenic neurotrypsin was assessed by Western blotting of agrin-90, which results from cleavage at both the α and the β site of agrin.

Transgenic mice with constitutive overexpression of neurotrypsin

To generate mouse lines with constitutive overexpression of active neurotrypsin conditional transgenic mice of line B6;C3-*Tg*(PRSS12) 491, 494 Zbz and B6.D2-*Tg*(Prss12) 533 Zbz were crossed with transgenic mice expressing Cre-recombinase under the control of the cytomegalovirus (CMV) promoter. The CMV promoter is active as early as in the embryonic two-cell to eight-cell stage embryo[637]. Therefore, loxP-based recombination results in a general deletion of the floxed segment and, thus, the germline transmission of the excised state. The constitutively transgenic mice were backcrossed for ten generations to C57BL/6 mice to produce the congenic strains B6.C3-*Tg*(PRSS12) 491.1 Zbz, B6.D2-*Tg*(Prss12) 533.1 Zbz and B6.C3-*Tg*(PRSS12) 494.1 Zbz. For convenience these mouse lines are referred to as Nto1, Nto2, and Nto3, respectively. Expression and proteolytic activity of the transgenic neurotrypsin were assessed by Western blotting (**Fig. 12a**, **19a**). An increase of agrin-90 in spinal cord homogenates of neurotrypsin-overexpressing mice was found.

Transgenic mice overexpressing inactive neurotrypsin

For a control transgenic mice overexpressing a catalytically inactive form of neurotrypsin under the same, i.e. the Thy1 promoter, were generated. Inactive neurotrypsin was generated by mutating the essential active site serine 711 (corresponding to serine 195 of chymotrypsin) to alanine. Because in all serine proteases, the active site serine is involved in a covalent intermediate of the proteolytic reaction, its mutation results in a complete loss of catalytic function. To generate the pThy1-neurotrypsin construct containing the serine-to-alanine mutation of the active site serine, a PCR fragment was amplified using mouse neurotrypsin cDNA as a template and 5'-CGTGTGGACAGCTGCCAGGGAGACGCTGGAGGA-3' and 5'-CTCAAGCTTAGTTACAGACTGGTGACACTTTTTATC-3' as primers. This fragment was reintroduced into full-length neurotrypsin. The mutated neurotrypsin cDNA was inserted into the *Xho*I site of the Thy1.2 cassette. The founders were backcrossed for ten generations to C57BL/6J to produce congenic strains. In the present study we used line B6.D2-*Tg*(Prss12(S711A)) 785 Zbz (Nto-inact). Overexpression of neurotrypsin and levels of agrin-90 in this line are shown in **Fig. 12a**. Quantification indicated that agrin-90 in these mice was at wild type levels (**Fig. 12d**).

Transgenic mice overexpressing cleavage-resistant agrin

The cDNA of the transmembrane form of rat agrin was subcloned into pBluescript. The arginine of the α cleavage site and the lysine of the β cleavage site were both mutated to alanine by site-directed PCR mutagenesis and thereby rendered resistant to neurotrypsin cleavage. The 5' end of the secreted form of agrin up to the first follistatin domain was obtained by RT-PCR using primers mNTAClaf (5'-CGCATCGATGTTCCGGGCTGCGCCATGGTCC-3') and mNTAr (5'-GTCCCGGGACCCACATGGCCCTTG-3') and total RNA from mouse spinal cord as template. The 5' region of transmembrane rat agrin upstream of the *Sma*I restriction site immediately following the DNA region encoding the first follistatin domain was then replaced by the 5' region of secreted murine agrin. The assembled DNA sequences of mutant (cleavage-resistant) and wild type agrin were inserted into the Thy1.2 expression cassette. The transgenic founders were backcrossed for ten generations with C57BL/6 mice to produce congenic strains. In this study, we used the line B6.D2-*Tg*(Agrn)1381Zbz (Ag) for overexpression of wild type agrin and the line B6.D2-*Tg*(Agrn(R995A;(K1754A))1385Zbz (rAg) for overexpression of cleavage-resistant agrin. The transgenic overexpression of agrin in neurons was confirmed by immunocytochemical staining (Bolliger et al., in preparation).

Double-transgenic mice overexpressing neurotrypsin and agrin

To overexpress neurotrypsin in combination with agrin, the constitutive neurotrypsin-overexpressing line B6.C3-*Tg*(PRSS12)491.1Zbz (Nto1) was crossed with mice of lines B6.D2-Tg(Agrn)1381Zbz (Ag) or mice of lines B6.D2-*Tg*(Agrn(R995A;(K1754A))1385Zbz (rAg).

Double transgenic offspring was referred to as Nto1/rAg and Nto1/Ag, respectively.

Aged mice

C57BL/6 mice were used for the aging analysis. Animals specified as aged were 24 months old, according to a median life expectation of about 31 months[460]. It was carefully paid attention that these animals did not suffer from unrelated age-associated diseases that might influence muscle morphology.

Mice overexpressing synaptophysin.

The synaptophysin-GFP (spGFP) transgenic mice have been previously described[638]. They were generated using the Thy1.2 expression cassette and had no detectable phenotype. The expression of the transgene did not affect the physiology (quantal content, post-tetanic potentiation and depression) and anatomy (ultrastructure of pre- and postsynaptic terminals, paralysis-induced nerve sprouting) of NMJs.

2.2 Antibodies

The goat antiserum G87 was raised against the protease domain of human neurotrypsin (produced in *E. coli*) and was purified by affinity chromatography. The rabbit antiserum R132 was raised against agrin90 of rat agrin (produced in HEK293T cells) and affinity-purified. The goat antiserum G92 was raised against agrin-22 (produced in HEK293T cells). Rabbit anti-synaptophysin antibodies were purchased from DakoCytomation, mouse anti-β-actin (clone AC-74) and anti-α-tubulin (clone DM 1A) from Sigma, rabbit anti-neural cell adhesion molecule (NCAM) and rabbit anti-$Na_v1.5$ from Chemicon, rabbit anti-phospho (Ser10)-acetyl (Lys14)-histone H3 (PAcH3) from Upstate, and rabbit anti-myogenin from Millipore. Secondary antibodies for immunoblotting (anti-rabbit, anti-goat, and anti-mouse peroxidase conjugates) were from Sigma. Secondary antibodies for immunofluorescence (donkey anti-rabbit Cy3 and Cy5, donkey anti-goat Cy5, and donkey anit-rabbit amino-methyl-coumarin-acetate (AMCA) conjugates) were from Jackson ImmunoResearch.

2.3 Electrophoresis and immunoblotting

Spinal cord extracts and muscle extracts were prepared by incubating the tissues in lysis buffer (150 mM NaCl, 1% Triton X-100, protease inhibitors, in 20 mM Tris-HCl, pH 7.5). After homogenization and centrifugation of the extracts, supernatants were collected and protein concentrations were measured by a Bradford assay (Bio-Rad Laboratories). Proteins were resolved by SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen) and blotted to PVDF membranes (Millipore). The membranes were dried (10 seconds incubation in methanol, air-drying for 15 minutes) and incubated for 90 min with primary antibodies in blocking solution (5% Roche Western blocking reagent, 0.1% Tween-20, in TBS). After washing with TBS containing 0.1% Tween-20, the blots were incubated with peroxidase-conjugated secondary antibodies for 60 min, and washed again. ChemiGlow (Alpha Innotech) was applied and immunoreactive bands were detected on a Fuji Imager LAS3000. For reprobing, membranes were incubated twice for 30 min in stripping solution (0.1% SDS, 1% Tween-20, in 200 mM glycine-HCl, pH 2.5). The signals were quantified using the open source NIH ImageJ image analysis software. All blots were performed in triplicates.

2.4 Grip strength and footprint analysis

Forelimb grip strength was measured using a standard device including a metal grip bar and

a tension spring balance. The animal was hold by the tail, allowed to grab the bar, and pulled constantly in one direction. The maximal strength until the mouse loosed the bar was recorded. Each animal was tested in 15 trials from which the 5 highest and lowest values were cancelled to minimize variations. This procedure was repeated at 3 consecutive days, resulting in 15 values per mouse, from which the average was calculated.
Footprint analysis was performed in a box with a small channel on an interchangeable sheet of paper. Fore- and hindpaws of a mouse were carefully labeled with black drawing ink (Pelikan). Most of the mice walked voluntarily and at constant speed through the channel to the other end of the box, thereby leaving their footprints. Mice that moved not voluntarily, stopped during the walk, changed their speed, jumped or ran at high speed were not included in the analysis. The footprints were digitized and processed using ImageJ. Fore- and hindpaw prints were discriminated by their distinct shapes. The stride length was measured as the averaged distance between all consecutive forepaw and consecutive hindpaw prints. Paw placement was quantified by measuring the distance between corresponding fore- and hindpaw prints.

2.5 Preparation of muscle cross-sections

Mice were euthanized by CO_2 inhalation and two skeletal muscles, musculus soleus and musculus extensor digitorum longus (EDL), were removed. The muscles were embedded in tissue-tek (Sakura) and frozen in isopentane cooled to -75 °C.
The muscles were placed in a cryostat (Leica CM3050) pre-cooled to -16 °C and serial 12 μm cross-sections were cut from the midbelly region of the muscles.

2.6 Skeletal muscle histochemistry

Muscle sections were either stained with hematoxylin and eosin (H&E) or for myofibrillar actomyosin ATPase (mATPase) activity. Before staining with H&E, sections were fixed in methanol for 5 minutes at -20°C. After washing, they were incubated for 10 minutes in hematoxylin solution according to Mayer (Fluka). After one minute of incubation in 70% ethanol containing several drops of HCl the sections were developed for three minutes under warm running water. Then, three minutes of counterstaining in 10 g/l Eosin Y (Sigma) was followed by dehydration in ethanol, clearing in xylene, and the embedding in eukitt quick-hardening mounting medium (Fluka). Staining for mATPase activity was performed after preincubation at pH 4.3 according to a modification of the procedure of Brooke and Kaiser [229,639]. Sections were incubated at room temperature for 7 min in a solution consisting of 54.3 mM sodium acetate and 32.6 mM sodium barbital, adjusted with HCI to pH 4.30. After washing with a solution containing 18 mM $CaCl_2$ and 100 mM Tris-HCI, pH 7.8, the sections were incubated at room temperature for 45 min in a medium containing 4.5 mM ATP, 19.5 mM $CaCl_2$, 116 mM 2-amino-2-methyl-l-propanol at pH 9.4. After three successive 3-min incubations in 11 mM $CaCl_2$, the sections were incubated two times for 1.5 min in 2% (w/v) $CoCl_2$ and then incubated six times for 30 sec in 10 mM sodium barbital and once in distilled water. After 45 sec of incubation in 2% (v/v) $(NH_4)_2S$, sections were washed in tapwater, dehydrated in ethanol, cleared in xylene, and embedded in eukitt quick-hardening mounting medium (Fluka).
Images were taken with a Leica DFC350FX digital camera that was mounted on a Leica DM-RXE inverted light microscope. Image analysis was performed using ImageJ.
H&E stained sections were used to determine the number of fibers, the cross-sectional area (CSA) of muscle and individual fibers, the fiber shape, and the amount of centrally nucleated fibers. For each muscle several cross-sections from the midbelly region of the muscle were used to

count the number of fibers and to measure the CSA. The biggest two sections per muscle were used for analysis, leading to four numbers per animal, from which the average was calculated. At least four animals in each group were compared. The CSA and the shape of the individual muscle fibers were determined by manually outlining all fibers in one muscle per animal using ImageJ. To assess the fiber shape, the circularity was used (circularity = 4π * area / perimeter^2). The circularity of a perfect circle is one and fibers deviating from a circle (i.e., those being more angular) exhibit a smaller area relative to their perimeter and, hence, their circularity is smaller than one. Centralized nuclei were counted on one section per animal and the percentage of fibers with centrally located nuclei was calculated. For all parameters, the average from at least four animals in each group was taken.
Cross-sections stained for mATPase activity were used for fiber typing. The proportion of darkly stained fibers was determined on two sections per muscle, leading to four numbers per animal, from which the average was taken. At least four animals in each group were used for the comparison. Fiber type grouping was analyzed using the method of enclosed fibers[267,640]. The number of type I and type II fibers at the boarder or inside of a muscle were counted and the number of enclosed type I and type II fibers (internal fibers that are surrounded by only the fibers of its own types) was determined. The formula defined by Lexell and Downham[267] was used to calculate T1 and T2 values. For each muscle two sections were analyzed, resulting in four T1 and T2 values per animal, from which the averages were calculated. At least four animals in each group were compared.

2.7 Immunostaining of soleus muscles

To analyze the NMJs, soleus muscles were dissected, rinsed in PBS, and fixed in methanol at -20°C for 10 min. The muscles were washed in PBS and teased into small bundles of fibers using thin forceps. After incubation in blocking solution (5% horse serum, 1% BSA, 1% Triton X-100, 0.1% sodium azide, in PBS) for 2 hr, different combinations of antibodies were added. The first antibodies were applied overnight at 4°C. After washing in PBS containing 0.5% Triton X-100, fluorescently-labeled secondary antibodies were added together with fluorophore-conjugated α-bungarotoxin for 4h at room temperature. To label presynaptic terminals, rabbit anti-synaptophysin antibodies were used, followed by Cy3-conjugated donkey anti-rabbit antibodies and Alexa Fluor 488-conjugated α-bungarotoxin (0.1 μg/ml, Molecular Probes). To label nerve endings, muscle of spGFP transgenic mice were incubated with rabbit anit-neurofilament antibodies followed by Cy5-conjugated donkey anti-rabbit antibodies and tetramethylrhodamine (TMR)-conjugated α-bungarotoxin (1 μg/ml, Sigma). For staining of agrin-90, muscles of spGFP transgenic mice were incubated with R132 followed by Cy5-conjugated donkey anti-rabbit antibodies and TMR-conjugated α-bungarotoxin (1 μg/ml). To stain agrin-90 and agrin-22 muscles were incubated with R132 and G92 followed by AMCA-conjugated donkey anti-rabbit antibodies, Cy5-conjugated donkey anti-goat antibodies, and TMR-conjugated α-bungarotoxin (1 μg/ml). After washing, samples were embedded in fluorescent mounting medium (DakoCytomation). Z-serial images were collected using a Leica SP1 or SP2 confocal laser scanning microscope using a 63x objective. Images from different laser channels were sequentially collected to avoid cross-talking. Only endplates oriented in the plane of the image area were used.
To quantify NMJ fragmentation, maximal projections of confocal stacks of the presynaptic synaptophysin and the postsynaptic α-bungarotoxin stainings were made. The image analysis program ImageJ was used to count the number of fragments (unconnected fluorescently labeled spots, which were bigger than 2 μm^2) per junction and to measure the size of the synaptic area (total fluorescently labeled area).
To detect denervated muscle fibers thin muscle cross-sections (12 μm) were fixed in methanol

at -20°C for 5 min, washed in PBS and incubated with antibodies against NCAM and $Na_v1.5$ in blocking solution overnight at 4°C. After washing in PBS containing 0.5% Triton X-100, the cross-sections were incubated with Cy3-conjugated donkey anti-rabbit antibodies for 4 hr at room temperature. After washing, samples were embedded in fluorescent mounting medium (DakoCytomation) and analyzed with a Leica DM-RXE fluorescent microscope. Staining for phosphorylated and acetylated histone H3 (PAcH3) was done on isolated muscle fibers. The soleus muscles were fixed in 4% PFA in PBS for 30 min and extensively teased into single muscle fibers. They were incubated with PAcH3 antibodies in blocking solution overnight at 4°C. After three washing steps in PBS/0.5% Triton X-100, Cy3-conjugated donkey anti-rabbit antibodies and the nucleic acid stain Hoechst (5 μg/ml, Invitrogen) were applied for 4 hr. After washing, the single muscle fibers were carefully mounted in fluorescent mounting medium (DakoCytomation), so that the overlapping of fibers was avoided, and analyzed under Leica DM-RXE fluorescent microscope.

2.8 Number of motoneurons

The spinal cords of two animals (i.e. a wild-type and a transgenic or an adult and an aged) were prepared in parallel and compared. They were dissected, aligned, embedded in tissue tek (Sakura) and frozen in liquid isopentane cooled to -70°C. Ten serial 12 μm sections were cut at four different position in the lumbar and sacral regions of the spinal cords. The sections were dried for 1 hour at room temperature and fixed in 4% PFA in PBS. After washing in PBS the sections were incubated for 10 minutes in cresyl violet staining solution (0.5% cresyl violet acetate solved in warm water containing 0.3% acetic acid). After rinsing in water, the sections were dehydrated in ethanol, cleared in xylene, and embedded in eukitt quick-hardening mounting medium (Fluka). The alpha motoneurons were identified according to Blondet et al.[641] by their location in the ventral horn of the cord, their size (cell body diameter ≥ 30 μm[358,363] and area ≥ 350 μm^2), and the presence of a clearly identifiable nucleus. They were counted through the ten serial sections at the four positions. Thereby, the motoneurons were followed through the sections and it was taken care to count every motoneuron only once. The number of motoneurons was compared between the two spinal cords, which were processed in parallel, and relative differences were calculated. This was repeated for at least five sets of animals and the average of the differences were compared. Images were taken with a Leica DFC350FX digital camera that was mounted on a Leica DM-RXE light microscope. Image analysis was performed using NIH ImageJ image analysis software.

2.9 Statistical analysis

All data is presented as means ± standard deviations (s.d.). Transgenic animals were always compared to their wild type littermates. Aged C57BL/6 mice were compared to adult animals from the same strain. n always referred to the number of animals.

Statistical values were calculated with SPSS. Data was checked for normal distribution using Shapiro-Wilk and one-sample Kolmogorov-Smirnov tests and for homoscedasticity (homogeneity of variance) using Levene's tests.

To compare means, two-tailed Mann-Whitney tests were used. Multiple comparisons (comparison of wt, Nto1, Nto1/rAg, Nto1/Ag animals) were performed with one-way ANOVAs (analysis of variance), followed by Tukey (homoscedasticity) or Tamhane's T2 (heteroscedasticity) post-hoc tests. For pairwise comparisons (number of motoneurons), Wilcoxon Signed Ranks tests were applied.

Significance levels were defined at 0.05, 0.01 and 0.001.

3 RESULTS

3.1 Transgenic overexpression of neurotrypsin in mice results in an increased agrin cleavage

To study the effect of agrin cleavage at the neuromuscular junction on structure and function of skeletal muscles we analyzed a series of transgenic mouse lines (Bolliger et al., **Fig. 11**). Two transgenic mouse lines overexpressing the agrin-cleaving serine protease neurotrypsin pan-neuronally (Nto1, Nto2) were generated, using a conventional overexpression cassette composed of the Thy 1 promotor[634] and the ORF of neurotrypsin. Distinct neurotrypsin overexpression level in the different lines, were thought to cause dose-dependent effects. Furthermore, mice overexpressing neurotrypsin exclusively in motoneurons (Nto-moto) were generated by adding a transcriptional stop sequence, flanked by two loxP sites, between the Thy 1 promotor and the ORF of neurotrypsin. For selective activation of the transgene, these mice were crossed with a mouse line expressing cre recombinase under control of the Hb9 promotor[14]. Hb9 is a transcription factor that is selectively and transiently expressed in precursors of motoneurons[642]. These mice were used to confirm that the motoneuronal neurotrypsin expression is sufficient to induce the neuromuscular phenotype and to exclude any influence of the central nervous system. A drawback of these animals was the variation in the level of neurotrypsin expression. The recombination to activate the transgene is thought to proceed differentially in every single motoneuron, leaving

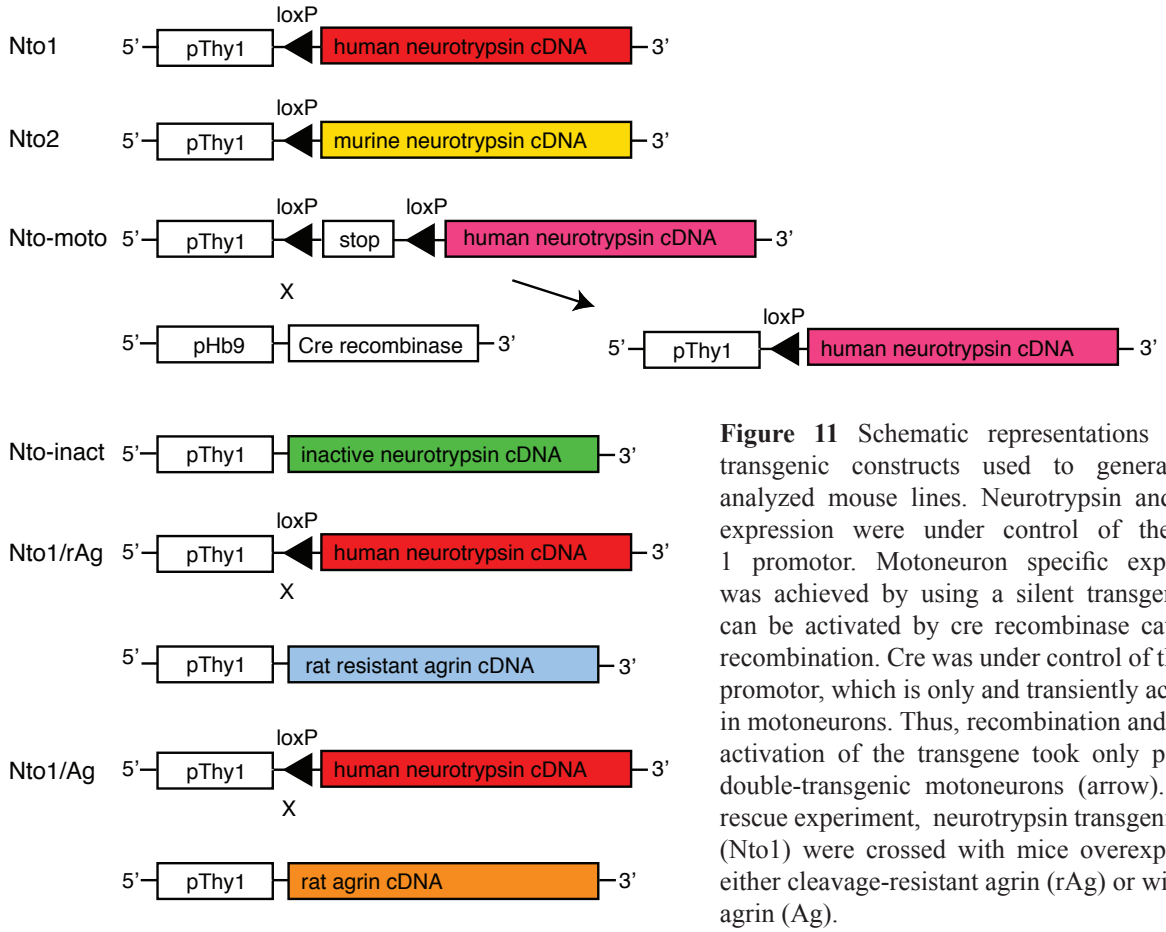


Figure 11 Schematic representations of the transgenic constructs used to generate the analyzed mouse lines. Neurotrypsin and agrin expression were under control of the Thy-1 promotor. Motoneuron specific expression was achieved by using a silent transgene that can be activated by cre recombinase catalyzed recombination. Cre was under control of the Hb9 promotor, which is only and transiently activated in motoneurons. Thus, recombination and hence, activation of the transgene took only place in double-transgenic motoneurons (arrow). For a rescue experiment, neurotrypsin transgenic mice (Nto1) were crossed with mice overexpressing either cleavage-resistant agrin (rAg) or wild type agrin (Ag).

different number of copies of the activated transgene behind. This process results in a substantial variation of the neurotrypsin expression level among motoneurons and among animals. A second drawback was the difficulty to perform a rescue experiment (see below). Therefore, most of the analysis was performed on line Nto1. Nto2 and Nto-moto were used to confirm the findings.

As a control, a mouse line overexpressing a catalytically inactive form of neurotrypsin, due to a mutation of the essential active site serine to alanine, was generated (Nto-inact). Mutation of the active site serine results in a complete loss of catalytic function in all serine proteases. This transgene was also expressed under control of the Thy 1 promotor. For a rescue experiment, neurotrypsin-overexpressing mice (line Nto1) were crossed with a mouse line overexpressing a

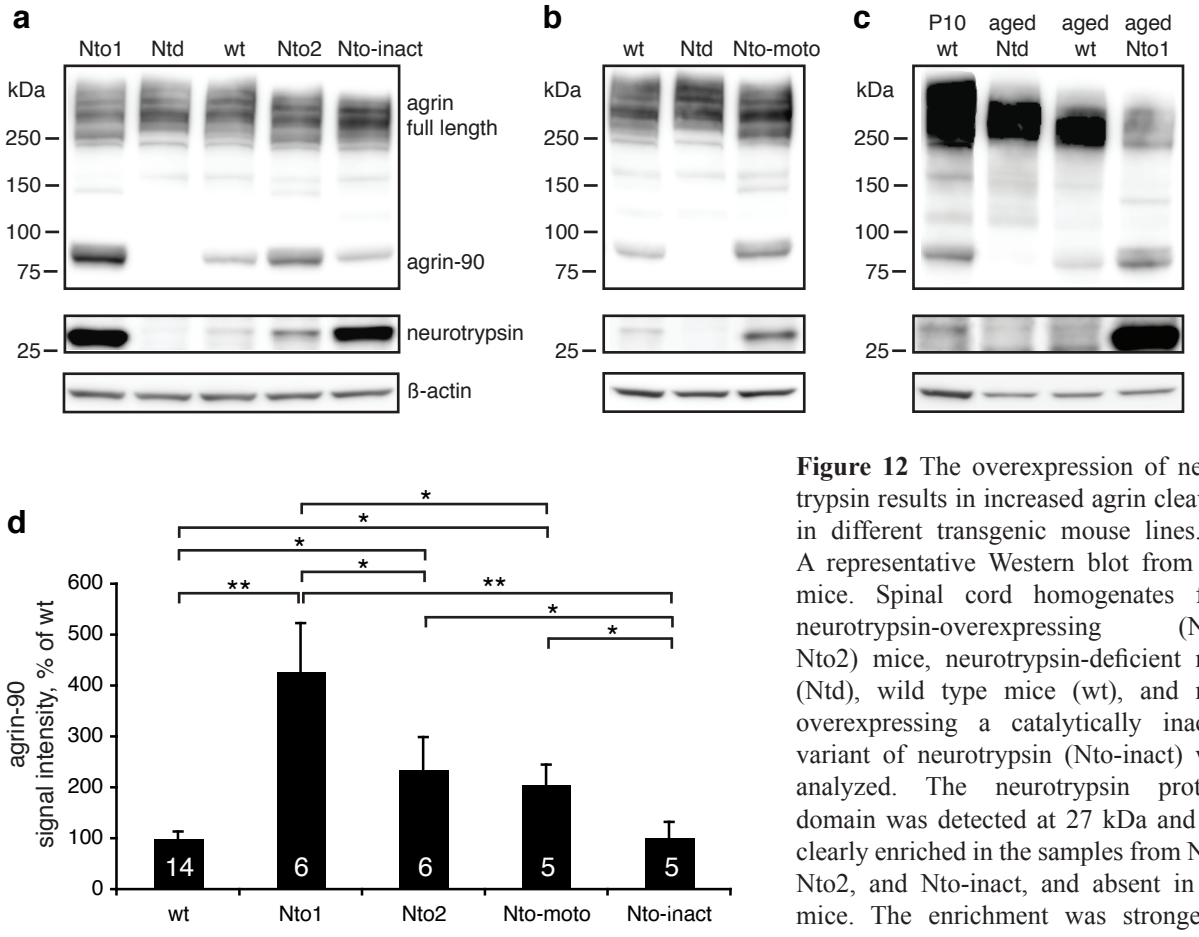


Figure 12 The overexpression of neurotrypsin results in increased agrin cleavage in different transgenic mouse lines. (**a**) A representative Western blot from P10 mice. Spinal cord homogenates from neurotrypsin-overexpressing (Nto1, Nto2) mice, neurotrypsin-deficient mice (Ntd), wild type mice (wt), and mice overexpressing a catalytically inactive variant of neurotrypsin (Nto-inact) were analyzed. The neurotrypsin protease domain was detected at 27 kDa and was clearly enriched in the samples from Nto1, Nto2, and Nto-inact, and absent in Ntd mice. The enrichment was stronger in Nto1 than in Nto2. Note that the protease domain of human neurotrypsin (overexpressed in Nto1) was slightly shifted towards lower molecular weight compared with mouse neurotrypsin (overexpressed in Nto2 and Nto-inact). The overexpression of neurotrypsin was associated with an increased agrin cleavage, which is represented by the accumulation of the agrin 90 kDa fragment (agrin-90), in the Nto1 and Nto2 mouse lines. Nto-inact did not show an increased immunoreactivity for agrin-90. Agrin-90 was completely absent in Ntd mice. ß-actin was used as loading control. (**b**) Increased signals for neurotrypsin and agrin-90 were also observed in mice overexpressing neurotrypsin exclusively motoneuronally (Nto-moto). (**c**) Representative Western blot from aged wt, Ntd, and Nto1 mice. Signals for neurotrypsin and agrin-90 were still detectable in aged wt, but their intensity was decreased compared to P10. The upper part of the agrin signal was absent in aged animals, regardless whether neurotrypsin was present or not. Note that neurotrypsin was still overexpressed in senescent Nto1 mice. (**d**) Quantitative analysis of the agrin-90 signals. A significant increase in the immunoreactivity for agrin-90 was detected in spinal cord homogenates from Nto1, Nto2, and Nto-moto P10 mice compared to wild type littermates (wild type values set to 100%). The signal intensity was higher in Nto1 than in Nto2 and Nto-moto mice. No difference was observed between Nto-inact and wild type mice. All Western blots were performed in triplicates. The number of animals for each group is indicated inside each bar. Error bars represent s.d. *P < 0.05, **P < 0.01, ***P < 0.001.

neurotrypsin-resistant agrin variant (rAg) in which the basic amino acids at P1 positions of the α and β cleavage sites were mutated to alanine. These mutations have been shown to completely abolish any cleavage by neurotrypsin[119]. Mice overexpressing neurotrypsin and a wild type agrin variant (Nto1/Ag) were used as negative control of the rescue experiment. Both agrin forms were overexpressed under control of the Thy 1 promotor. The rescue experiment with the Nto-moto mice was not performed, because the triple crossing (Nt/cre/rAg) would have demanded a high number of animals and would have been very time consuming. Neurotrypsin deficient mice (Ntd), in which the protease domain has been deleted, were also available. To compare several neuromuscular aspects of neurotrypsin transgenic with those of aged mice, aged wild-type mice were also investigated. Initial results indicated similarities, possibly leading to insights into the age-associated neuromuscular degeneration referred to as sarcopenia. Mice specified as adult were always 4 months old, whereas animals referred to as aged or senescent were 24 months old. For most of the analysis, female animals were used. Males were only occasionally analyzed to confirm the most important findings.

Neurotrypsin overexpression and the neurotrypsin-dependent agrin cleavage were verified by Western blotting of spinal cord homogenates of P10 mice (**Fig. 12a, b**). The G87 antibody was used to detect the neurotrypsin protease domain. This domain represents the activated form of neurotrypsin, after activation cleavage at the zymogen activation site. SDS-Page under reducing conditions was applied to separate the protease domain from the non-catalytic part and consecutive immunoblotting resulted in a signal at 27 kDa. An increased immunoreactivity for the neurotrypsin protease domain was detected in all transgenic mice compared to their wild type littermates. The different lines showed distinct expression levels (Nto1 > Nto2 and Nto-moto). The signal for the neurotrypsin protease domain was completely absent in the neurotrypsin deficient mice. Overexpression of active neurotrypsin was associated with increased agrin cleavage. A fragment of about 90 kD (agrin-90), which results from specific proteolytic cleavage of agrin at its α and β cleavage sites[119,121], was detected with the R132 antibody. All mouse lines overexpressing an active form of neurotrypsin showed an increased immunoreactivity for agrin-90. Quantification revealed not only significant increases in agrin-90 levels for all transgenic mouse lines, but also distinct agrin-90 amounts in the different lines (**Fig. 12d**). The enrichment of agrin-90 was in good correlation with the neurotrypsin overexpression level in different transgenic mouse lines (Nto1 > Nto2 and Nto-moto). This dose-dependency of agrin cleavage indicated that neurotrypsin might indeed processes agrin *in vivo*. Overexpression of proteolytically inactive neurotrypsin did not affect agrin cleavage, demonstrating that the proteolytic activity was responsible for enhanced agrin processing in neurotrypsin-overexpressing mice. Consistent with previous results, agrin-90 was completely absent in neurotrypsin deficient mice. These findings indicated that neurotrypsin could cleave agrin *in vivo*, and that its overexpression resulted in an excessive agrin cleavage in different transgenic mouse lines.

Western blotting on aged spinal cord extracts indicated that neurotrypsin was still expressed in senescence, although the weak signal for the neurotrypsin protease domain was only marginally over background levels seen in Ntd mice (**Fig. 12c**). The signal for agrin-90 was clearly increased in aged wild type mice compared to Ntd mice. Thus, neurotrypsin expression and agrin cleavage were sustained in senescence. However, the levels of both neurotrypsin and agrin-90 were decreased compared to 10 days old animals. Interestingly, the upper part of the signals for full length agrin, representing the most heavily glycanated variants[121], was absent in aged animals. This could represent increased cleavage or decreased synthesis of these agrin forms. Neurotrypsin appeared to be dispensable for this process, since the heaviest agrin variants were also absent in aged Ntd animals.

3.2 Neurotrypsin-overexpressing and aged mice show motor dysfunction

The transgenic mice overexpressing active neurotrypsin in neurons were viable. However, after developing normally during the first week of life, they began to lag behind their normal littermates with regard to growth of body mass. In adult animals of line Nto1 body weight dropped 9% following neurotrypsin overexpression (**Table I**). Transgenic animals moved around slowly, with small steps. Their gait was insecure and hesitant, with frequent stops, during which they assumed a resting position with spread legs. However, they're behavior, physical activity and food uptake appeared to be normal and they reached the same age as wild type littermates. In order to assess motor coordination of transgenic mice, their footprints were recorded (**Fig. 13a**). Quantification of stride length and paw placement revealed significant differences between transgenic mice and their wild type littermates. Stride length was reduced in transgenic mice and they placed their hindpaw at some distance instead of immediately behind their forepaw (**Fig. 13e**). Mice overexpressing neurotrypsin selectively in motoneurons (Nto-moto) exhibited similar deficits, suggesting a motoneuronal or muscular rather than a central mechanism (**Fig. 13b**). Since muscular deficits could result in decreased muscular strength, the forelimb grip strength was measured. Indeed, it was reduced by more than 10% in transgenic animals when compared with wild type littermates (**Fig. 14**). Thus, not only coordination, but also strength was affected. Mice overexpressing catalytically inactive neurotrypsin (Nto-inact) and double transgenic mice overexpressing neurotrypsin together with cleavage-resistant agrin (Nto1/rAg) were normal in regard to body weight, motor coordination and grip strength, whereas mice concomitantly overexpressing neurotrypsin and wild type agrin (Nto1/Ag) were not (**Fig. 13a, c, e, 14**). Thus,

Table I
Body and muscle masses in female mice

line	genotype	n	body mass (g)	soleus muscle mass (mg)	EDL muscle mass (mg)	muscle mass per body mass (mg/g)
Nto1	wt	26	24.2 ± 2.3	8.50 ± 0.59	9.62 ± 0.76	0.76 ± 0.04
	Nt	28	22.0 ± 1.7 **	7.55 ± 0.45 ***	8.94 ± 0.67 **	0.75 ± 0.05
x rAg	Nt/rAg	9	24.2 ± 1.6 °	8.50 ± 0.89 °°°	9.64 ± 0.66 °	0.75 ± 0.03
x Ag	Nt/Ag	11	22.0 ± 0.9 ***	7.40 ± 0.34 ***	8.88 ± 0.56 *	0.74 ± 0.04
Nto-inact	wt	5	24.6 ± 1.3	8.50 ± 0.69	9.89 ± 0.58	0.75 ± 0.03
	iNt	5	23.7 ± 2.9	8.37 ± 0.87	9.46 ± 1.06	0.75 ± 0.02
	adult	11	24.1 ± 3.4	8.72 ± 0.99	9.66 ± 1.06	0.76 ± 0.03
	aged	9	41.2 ± 7.2 ***	8.75 ± 0.67	8.73 ± 0.61 *	0.43 ± 0.07 ***

Both body mass and muscle mass were reduced in female animals overexpressing active neurotrypsin. Note that the loss of soleus muscle mass was more pronounced than the loss of EDL muscle mass. Concomitant overexpression of neurotrypsin-resistant agrin and neurotrypsin restored the wild type situation, wild type agrin did not. Aged animals exhibited increased body mass, no change in soleus muscle mass and a small reduction in EDL mass. n indicates the number of animals for each group. Data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °P < 0.05, °°P < 0.01, °°°P < 0.001 compared to Nto1.

proteolytic processing of agrin at the α and β cleavage sites by neurotrypsin was responsible for reduced body weight and motor deficits.

Aged wild type mice exhibited remarkably similar motor deficits. Their gait was insecure and hesitant. They took small steps and placed their hindpaw at some distance rather than immediately behind the forepaw (**Fig. 13d, e**). Their forelimb grip strength was significantly reduced, when compared with adult animals (**Fig. 14**). Body mass, however, was not reduced but significantly increased in aged animals, which was to a great extent attributable to an increase in body fat (**Table I**).

3.3 Excessive agrin cleavage results in morphological changes of the skeletal muscle similar to aging

To better understand the pathogenic process resulting in motor deficits in neurotrypsin-overexpressing mice, the skeletal muscle morphology was studied. A slow (m. soleus) and a fast twitch (m. extensor digitorum longus, EDL) muscle of adult mice were analyzed for alterations following neurotrypsin overexpression and excessive agrin cleavage. In concert with functional impairment and reduction in body weight, muscle mass was significantly reduced in neurotrypsin-overexpressing animals compared to wild type littermates. In line Nto1, wet weight of soleus and EDL muscle were reduced 11% and 7%, respectively (**Table I**). Additional overexpression of resistant agrin rescued from the neurotrypsin-induced muscle mass loss, whereas wild type agrin did not. Loss of body mass, muscle strength and muscle mass all were of similar magnitude and caused by enhanced agrin cleavage at the neurotrypsin specific α and β cleavage sites. Thus, at first glance, the loss of muscle

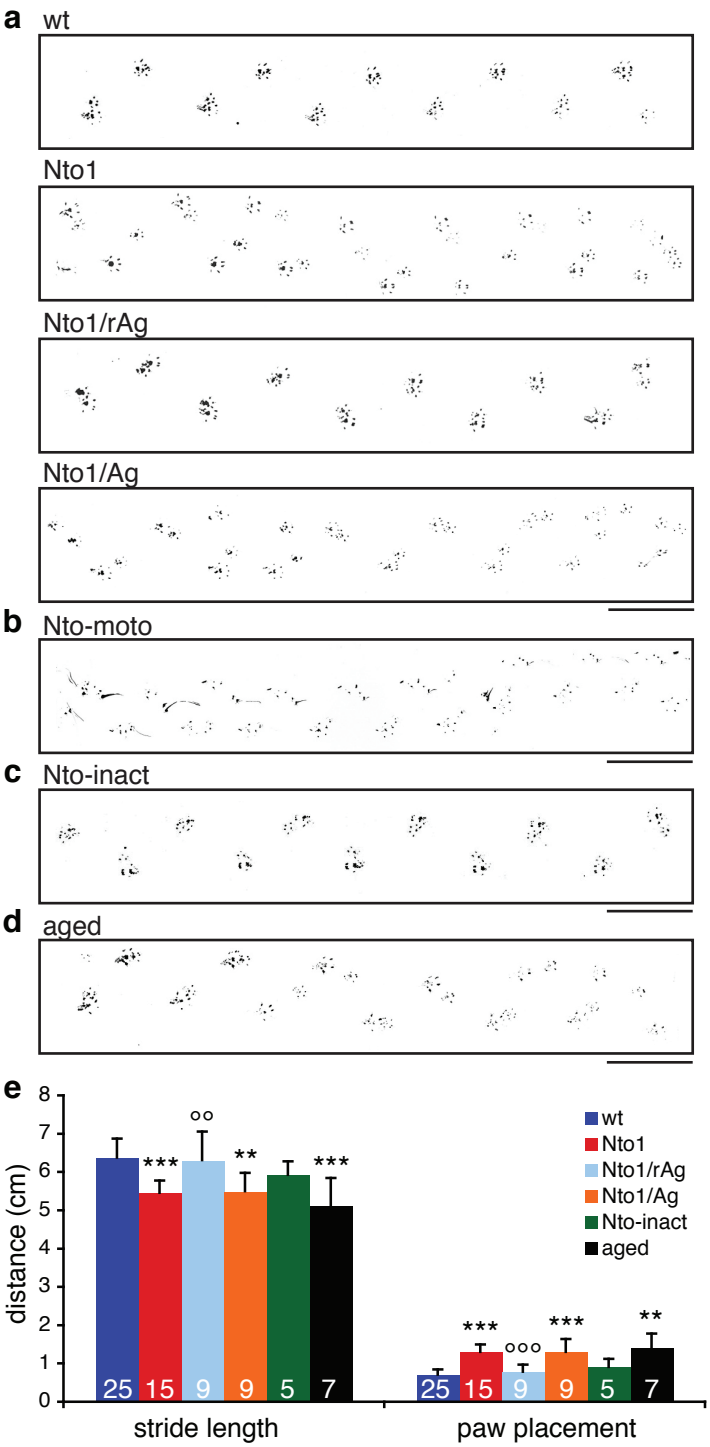


Figure 13 Neurotrypsin overexpression and enhanced agrin cleavage induces aging-like motor impairments. (**a-d**) Footprints recorded from Nto1 (**a**), Nto-moto (**b**), Nto-inact (**c**) and aged mice (**d**). Animals overexpressing active neurotrypsin as well as aged animals took small steps and exhibited an insecure gait. In contrast to wild type animals, they did not place their fore- and hindpaws at the same spot. Concomitant overexpression of cleavage-resistant neurotrypsin rescued from the motor coordination impairment, whereas wild type agrin did not. (**e**) Quantification of stride length and paw placement. Stride length was defined as the distance between consecutive fore- and hindpaw prints. Paw placement was quantified by measuring the distance between corresponding hind- and forepaw prints. Neurotrypsin overexpression and aging resulted in reduced stride length and impaired paw placement. The overexpression of neurotrypsin-resistant agrin rescued from the motor phenotype, wild type agrin did not. Inactive neurotrypsin had no effect. The number of animals for each group is indicated inside each bar. Error bars represent s.d. Scale bars, 5 cm. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to wild type littermates or adult controls, $^{°°°}P < 0.001$ compared to Nto1.

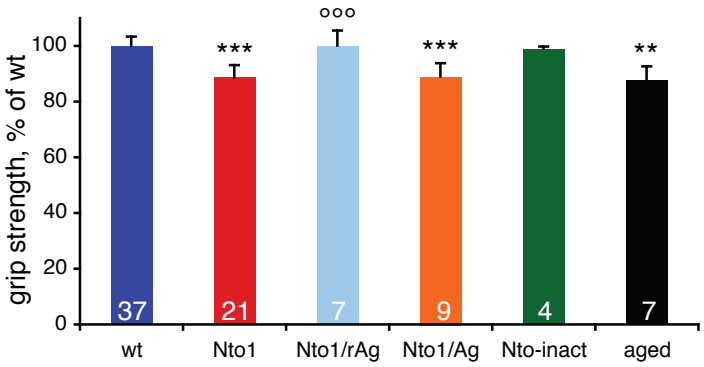


Figure 14 Forelimb grip strength was measured in 15 trials at each of 3 consecutive days. The strength was reduced in neurotrypsin-overexpressing and in aged mice compared to adult wild types (wt values set to 100%). Overexpression of resistant agrin together with neurotrypsin restored the wild type situation, wild type agrin did not. Inactive neurotrypsin did not affect forelimb grip strength. The number of animals for each group is indicated inside each bar. Error bars represent s.d. **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °°°P < 0.001 compared to Nto1.

mass and strength may simply be a consequence of reduced body mass. However, impairment of motor coordination implies a more complex disturbance.

Indeed, quantitative analysis of the number of fibers in hematoxylin and eosin stained muscle cross sections revealed a dramatic loss of muscle fibers in conditions with enhanced agrin cleavage (**Fig. 15**). This effect was observed in female (**Table II**) and male mice (**Table III**). The number of myofibers did not differ between sexes, neither for wild type nor for transgenic animals. All transgenic animals overexpressing an active form of neurotrypsin suffered from the fiber loss, although the severity varied considerably. Up to 30 % of all fibers of the soleus muscle are lost in line Nto1, only about 10 % in line Nto2. Qualitatively the same effect was observed in the EDL muscle. In agreement with the reduction in muscle mass, slow-twitch soleus was more affected than fast twitch EDL muscle. The severity of the fiber loss was consistent with the level of neurotrypsin overexpression and the amount of agrin cleavage, both

Table II
Number of fibers in soleus and EDL muscles of female mice

		m. soleus			m. edl		
line	genotype	n	number of fibers	rel. wt	n	number of fibers	rel. wt
Nto1	wt	24	932 ± 56		15	1008 ± 51	
	Nt	21	651 ± 50 ***	0.70	11	865 ± 55 ***	0.86
x rAg	Nt/rAg	9	929 ± 48 °°°	1.00	8	969 ± 31 °°°	0.96
x Ag	Nt/Ag	10	637 ± 49 ***	0.68	7	855 ± 73 ***	0.85
Nto2	wt	4	939 ± 21		4	1031 ± 32	
	Nt	6	861 ± 29 *	0.92	4	907 ± 76 *	0.88
Nto-moto	wt	9	941 ± 40		5	1002 ± 51	
	Nt/cre	6	535 ± 43 **	0.57	5	715 ± 64 **	0.71
Nto-inact	wt	7	942 ± 23		5	977 ± 62	
	iNt	7	952 ± 38	1.01	5	979 ± 42	1.00
Ntd	wt	6	952 ± 55		6	988 ± 82	
	ko	7	943 ± 37	0.99	6	1007 ± 40	1.02
	adult	10	960 ± 30		7	1000 ± 51	
	aged	15	813 ± 63 ***	0.85	6	824 ± 37 **	0.82

Enhanced agrin cleavage and aging are associated with a loss of muscle fibers. The number of fibers of soleus and EDL muscles of female mice were counted on muscle cross-sections. All animals were compared to wild type littermates. The overexpression of active neurotrypsin was associated with a loss of muscle fibers in both soleus and EDL muscles. Overexpression of cleavage-resistant but not wild type agrin rescued from the fiber loss. Note that the loss of fibers was more pronounced in line Nto1 than in line Nto2. Motoneuron-specific neurotrypsin overexpression was sufficient to induce the effect. Inactive neurotrypsin or the absence of neurotrypsin did not affect the muscle fiber number. Aging also resulted in a loss of muscle fibers in both soleus and EDL muscle. n indicates the number of animals for each group. Data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °°°P < 0.001 compared to Nto1.

being stronger in line Nto1 than in line Nto2. A higher neurotrypsin expression level resulted in an increased agrin cleavage and in more extensive muscle fiber loss. This dose dependency indicated that neurotrypsin overexpression and enhanced agrin cleavage could induce the muscle fiber loss. The strongest phenotype was observed in Nto-moto mice, which lost more than 40% of their myofibers. A direct comparison of the neurotrypsin expression level and the amount of agrin cleavage to the other lines was not possible, due to the motoneuron-restricted expression pattern. However, the severe phenotype of these animals suggested that the motoneuronal neurotrypsin overexpression was sufficient and especially important to induce muscle fiber loss. The overexpression of inactive neurotrypsin did not affect the number of fibers, neither in the soleus nor in the EDL muscle, proving that the proteolytic activity of neurotrypsin was

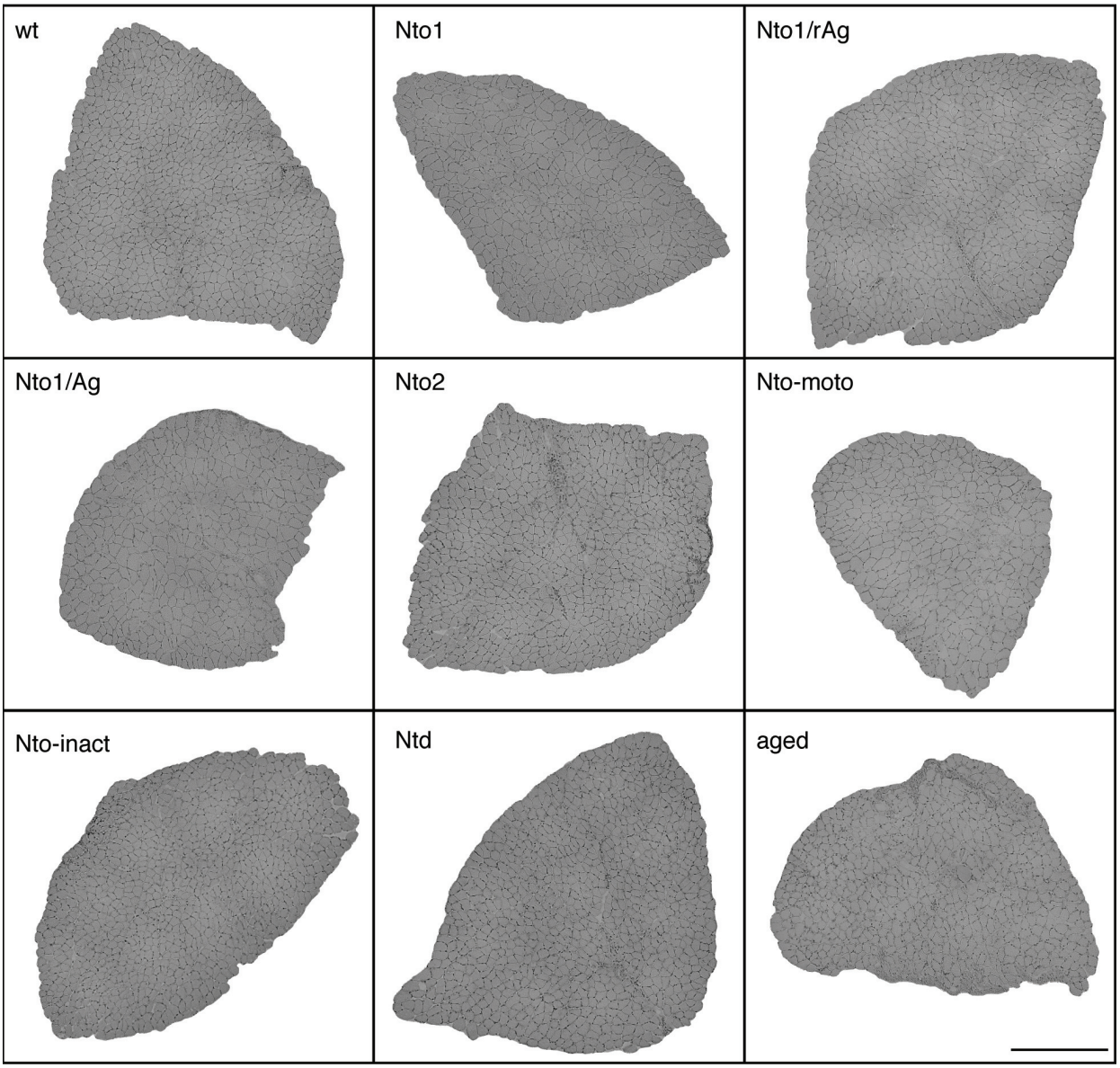


Figure 15 Muscle thickness and fiber number is reduced in conditions with enhanced agrin cleavage. Representative hematoxylin and eosin (H&E) stained cross-sections from soleus muscles illustrate reduced muscles thickness and fiber number in female mice from line Nto1 and Nto-moto. Concomitant overexpression of a cleavage-resistant agrin variant and neurotrypsin restored the wild type muscle morphology, whereas wild type agrin did not. Animals from line Nto2 and aged animals only exhibited minor changes in muscle thickness, but the number of fibers appears to be reduced. Cross-sections from Nto-inact and Ntd mice were not distinguishable from wild type sections. Scale bar, 0.5 mm.

Table III
Number of fibers in soleus and EDL muscles of male mice

		m. soleus			m. edl		
line	genotype	n	number of fibers	rel. wt	n	number of fibers	rel. wt
Nto1	wt	14	952 ± 39		6	1002 ± 48	
	Nt	12	644 ± 46 ***	0.68	5	839 ± 37 **	0.84
x rAg	Nt/rAg	4	931 ± 22 °°°	0.98			
x Ag	Nt/Ag	4	706 ± 46 ***	0.74			
Nto2	wt	4	973 ± 12				
	Nt	5	871 ± 25 *	0.90			
Nto-moto	wt	7	977 ± 38		4	1001 ± 23	
	Nt/cre	4	546 ± 58 **	0.56	2	757 ± 36	0.76
Nto-inact	wt	3	942 ± 14				
	iNt	5	945 ± 50	1.00			
Ntd	wt	3	923 ± 18				
	ko	4	974 ± 20	1.05			
	adult	10	948 ± 27		6	1008 ± 44	
	aged	8	870 ± 32 ***	0.92	4	858 ± 119 *	0.85

Neurotrypsin and aging induced loss of muscle fibers was also observed in male muscles. n indicates the number of animals for each group. Data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °°°P < 0.001 compared to Nto1.

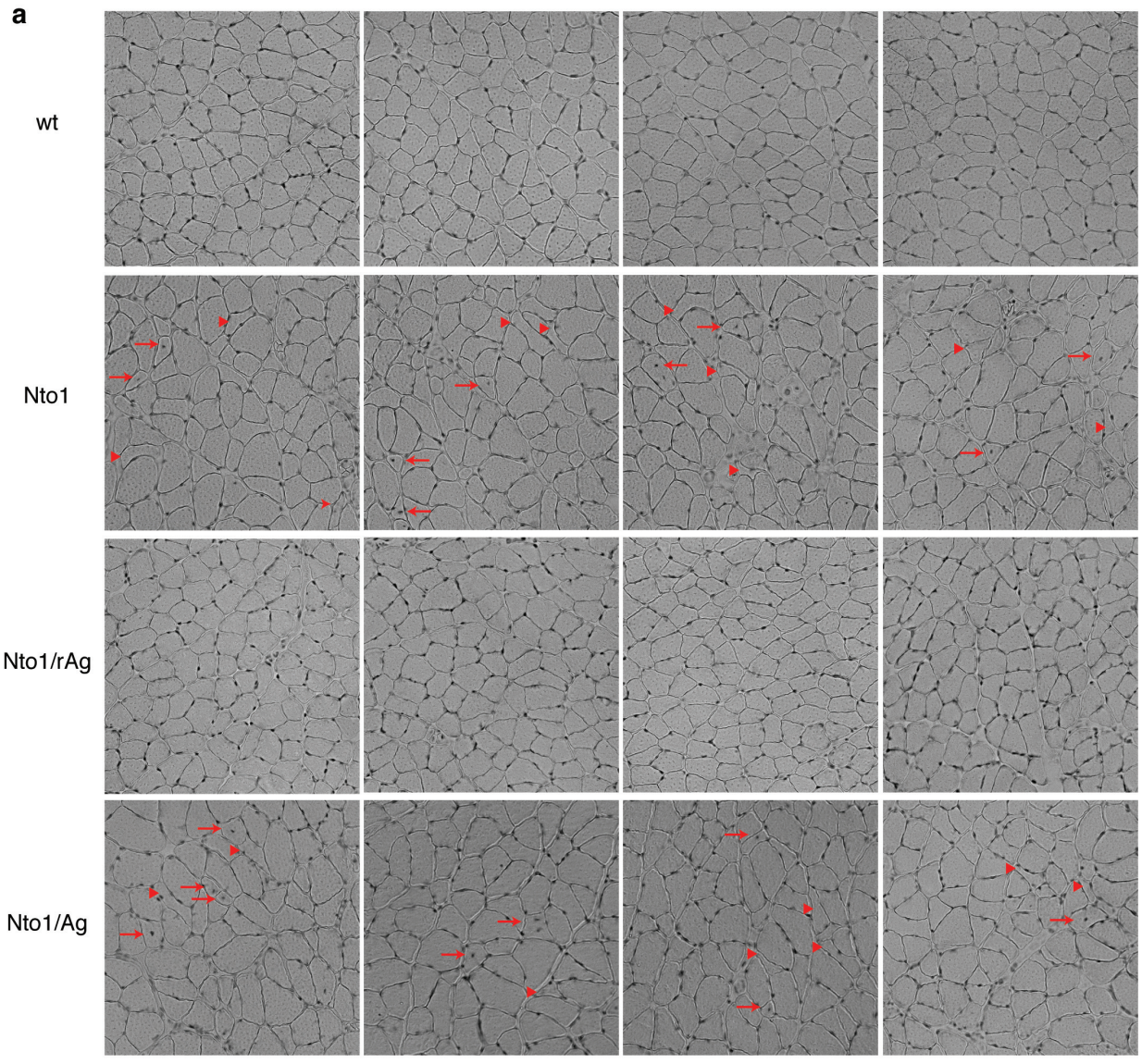
needed. Furthermore, the concomitant overexpression of a neurotrypsin-resistant agrin variant and neurotrypsin (Nto1/rAg) rescued the fiber loss in both soleus and EDL muscle. This was not the case if a wild type agrin variant (Nto1/Ag) was used (**Table II**, **Fig. 15**). Therefore, cleavage of agrin at the neurotrypsin-specific α and β cleavage sites was necessary to establish the phenotype. The absence of neurotrypsin, in contrast, did not cause any change in the number of muscle fibers in Ntd mice.

Table IV
Cross-sectional areas (CSAs) of the whole soleus muscle and individual muscle fibers

		muscle CSA (mm^2)			fiber CSA (μm^2)		
line	genotype	n		rel. wt	n		rel. wt
Nto1	wt	24	1.58 ± 0.16		15	1391 ± 100	
	Nt	21	1.41 ± 0.19 **	0.89	12	1773 ± 178 ***	1.27
x rAg	Nt/rAg	9	1.64 ± 0.17 °°	1.04	7	1437 ± 138 °°°	1.03
x Ag	Nt/Ag	10	1.38 ± 0.10 *	0.88	7	1838 ± 155 ***	1.32
Nto2	wt	4	1.61 ± 0.14		4	1349 ± 81	
	Nt	6	1.53 ± 0.07	0.95	4	1550 ± 80 *	1.15
Nto-moto	wt	9	1.63 ± 0.14		5	1408 ± 85	
	Nt/cre	6	1.35 ± 0.17 *	0.83	5	2241 ± 250 **	1.59
Nto-inact	wt	7	1.60 ± 0.07		5	1372 ± 29	
	iNt	7	1.57 ± 0.07	0.98	5	1392 ± 95	1.02
Ntd	wt	6	1.57 ± 0.04		5	1364 ± 113	
	ko	7	1.56 ± 0.09	0.99	6	1354 ± 116	0.99
	adult	10	1.63 ± 0.12		6	1410 ± 95	
	aged	15	1.54 ± 0.09	0.95	7	1597 ± 76 **	1.13

Reduction in muscle thickness was accompanied by an increase in mean fiber thickness in female animals from neurotrypsin-overexpressing mouse lines Nto1, Nto2, and Nto-moto. Note that the effects were more pronounced in line Nto2 than in line Nto1. Motoneuronal neurotrypsin overexpression was sufficient to induce both reduction in muscle CSA and increase in mean fiber CSA. Cleavage-resistant agrin restored the wild type situation, wild type agrin did not. Aged soleus muscles also tended to be thinner and to consist of thicker muscle fibers. n indicates the number of animals for each group. All fibers of each muscle were used for quantification. Data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °°P < 0.01, °°°P < 0.001 compared to Nto1.

To examine the influence of muscle fiber loss on muscle thickness, the cross-sectional area (CSA) of the soleus muscle was measured (**Table IV**, **Fig. 15**). It was reduced in all mice overexpressing active neurotrypsin compared to their wild-type littermates. Reduced muscle thickness was most likely a consequence of the fiber loss and the cause for reduced muscle strength. Surprisingly, reduction in muscle CSA was clearly less pronounced than the loss of fibers, attributable to a significant increase in the CSA of the individual muscle fibers (**Table IV**). However, measurements of individual fiber thicknesses revealed not a general increase, but a greater heterogeneity of the fiber CSAs. Both the fraction of atrophied fibers and the fraction of hypertrophied fibers were significantly increased in neurotrypsin-overexpressing animals (**Fig. 16, 17**). This finding suggests that not all fibers are equally affected. The loss and atrophy of certain fibers may be partially compensated by the hypertrophy of unaffected fibers. The increase of the mean fiber thickness could be a compensatory mechanism to the fiber loss, keeping the animal mobile. The whole muscle atrophy, the increase in mean fiber CSA and the greater fiber size variability was observed in all line overexpressing active neurotrypsin. Also here, the extent of the effects was consistent with the level of neurotrypsin and agrin cleavage, all being stronger in line Nto1 than in line Nto2 (**Table IV, Fig. 16b, Fig. 17**). The strong phenotype in Nto-moto mice indicated that neurotrypsin overexpression in motoneurons sufficiently explained the observed phenotype



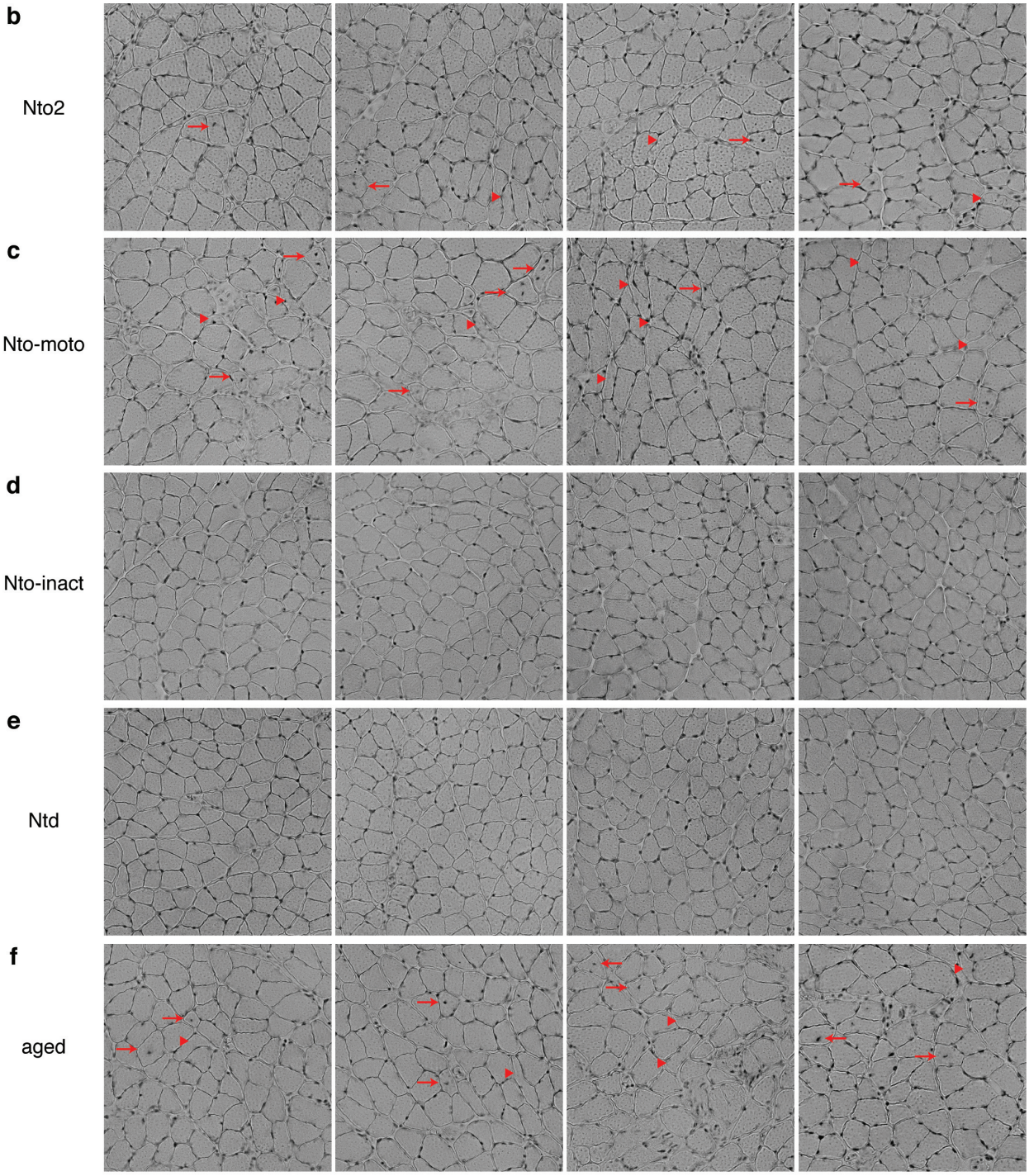


Figure 16 Similar changes of the skeletal muscle morphology due to neurotrypsin overexpression and aging. (**a**) H&E stained cross-sections from soleus muscle of female mice overexpressing neurotrypsin or neurotrypsin together with cleavage-resistant or wild type agrin. Increased heterogeneity of fiber thickness, centralized nuclei (arrows) and angular fibers (arrowheads) were observed in conditions with enhanced agrin cleavage (Nto1, Nto1/Ag). Muscles of Nto1/rAg mice were not distinguishable from those of wild type littermates. (**b**) Cross-sections from Nto2 muscles showed similar but weaker alterations of the muscle morphology. (**c**) Motoneuronal neurotrypsin overexpression was sufficient to induce heterogeneity of muscle thickness, as well as the accumulation of angular and centrally nucleated muscle fibers. (**d, e**) The overexpression of an inactive neurotrypsin form and the absence of neurotrypsin did not affect the skeletal muscle morphology. (**f**) Cross-sections from aged mice were strikingly similar to those from neurotrypsin transgenic mice. Greater heterogeneity of fiber thickness, centralized nuclei, and angular fibers can be observed upon both excessive agrin cleavage and aging. Scale bars, 0.1 mm.

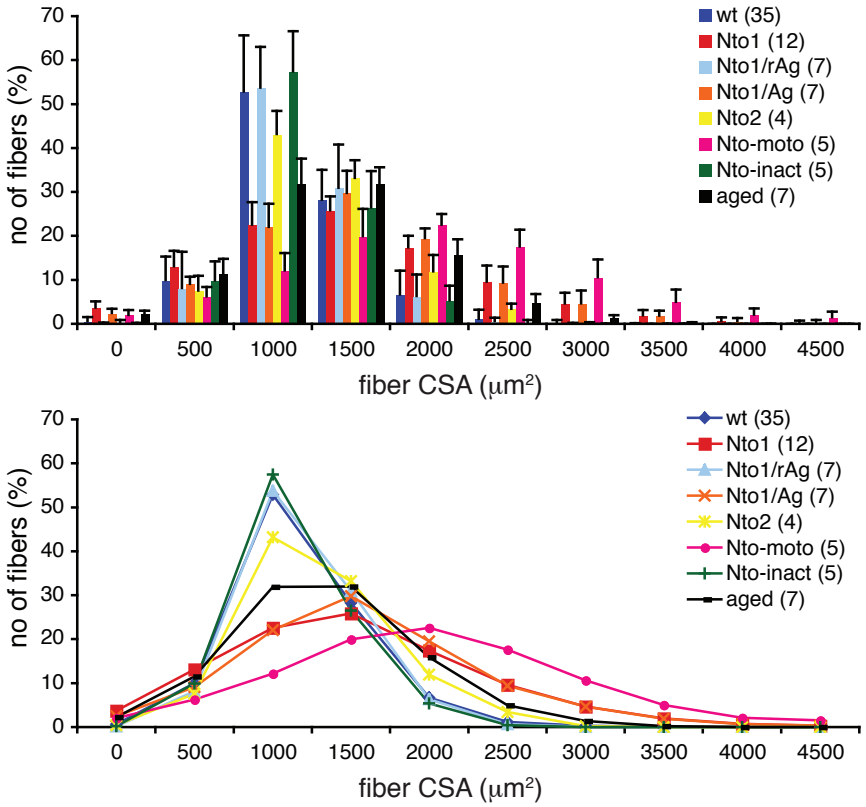


Figure 17 Increased heterogeneity of fiber thickness is a common feature of neurotrypsin transgenic and aged muscles. Frequency histogram of fiber thickness in soleus muscles from Nto1, Nto2, Nto-moto, Nto-inact, and aged mice. Note the accumulation of atrophied and hypertrophied muscle fibers in mice overexpressing active neurotrypsin and in aged mice. The effect was clearly weaker in line Nto2 than Nto1. Cleavage-resistant agrin restored the wild type situation, wild type agrin did not. Inactive neurotrypsin did not induce fiber size variability.
All fibers of each muscle were measured for the quantification. The number of animals for each group is indicated in parentheses. Error bars represent s.d

(**Fig. 16c**). Line Nto-inact did not show any fiber size alterations, outlining the importance of the proteolytic activity (**Fig. 16d**). The overexpression of a neurotrypsin-resistant agrin variant together with neurotrypsin rescued the fiber size alterations, whereas the overexpression of a wild type agrin variant did not. The cleavability of agrin was again crucial to induce the phenotype.
Beside the number and the size of the myofibers, their shape was analyzed on soleus muscle cross-sections. The circularity, which is a measure of the compactness of a shape, was used. It describes the ratio of the perimeter to the area. The circularity of a perfect circle is one and more angular fibers exhibit smaller circularities. The mean circularity of the soleus muscle fibers was significantly decreased in conditions with enhanced agrin cleavage (**Table V**). Consistently, angular fibers, which are characterized by a low circularity, appeared (**Fig. 16, 18**). Because angular fibers are considered to be indicators of denervation[158,197], their appearance suggested ongoing denervation of muscle fibers. Denervation is known to induce severe muscle atrophy, indicating that the loss of neuronal input may be responsible for both the accumulation of atrophied fibers

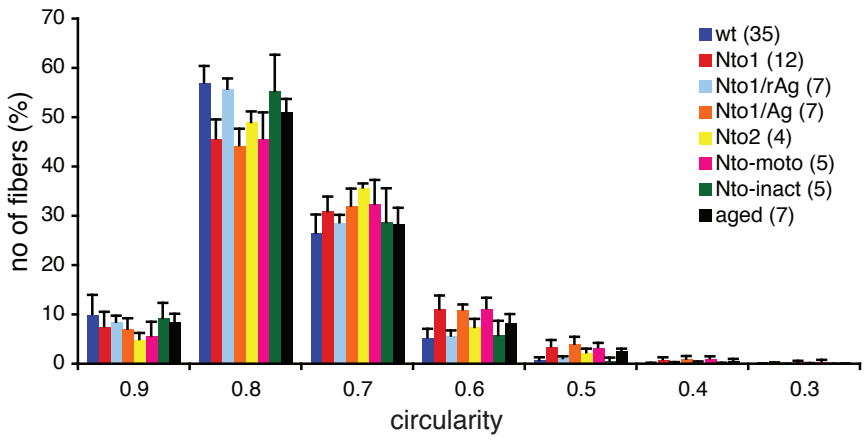


Figure 18 Appearance of angular fibers in conditions with enhanced agrin cleavage and in senescence. Frequency histogram of the fiber circularity in soleus muscles showed that the amount of angular fibers (low circularity) was increased due to overexpression of active neurotrypsin and aging.
All fibers of each muscle were analyzed. The number of animals for each group is indicated in parentheses. Error bars represent s.d.

Table V
Central nucleation and circularity of soleus muscle fibers

		centrally nucleated fibers (%)			fiber shape (circularity)		
line	genotype	n			n		
Nto1	wt	15	0.44 ± 0.11		15	0.819 ± 0.011	
	Nt	12	2.94 ± 0.76	***	12	0.789 ± 0.013	***
x rAg	Nt/rAg	7	0.40 ± 0.10	°°°	7	0.815 ± 0.005	°°°
x Ag	Nt/Ag	7	2.41 ± 0.54	***	7	0.785 ± 0.011	***
Nto2	wt	4	0.34 ± 0.18		4	0.817 ± 0.010	
	Nt	4	0.79 ± 0.11	*	4	0.795 ± 0.007	*
Nto-moto	wt	5	0.41 ± 0.10		5	0.828 ± 0.004	
	Nt/cre	5	2.23 ± 0.59	**	5	0.783 ± 0.015	**
Nto-inact	wt	5	0.48 ± 0.15		5	0.816 ± 0.013	
	iNt	5	0.48 ± 0.12		5	0.817 ± 0.017	
Ntd	wt	5	0.41 ± 0.20		5	0.824 ± 0.006	
	ko	6	0.45 ± 0.31		6	0.821 ± 0.007	
	adult	6	0.45 ± 0.14		6	0.821 ± 0.011	
	aged	7	1.88 ± 0.86	**	7	0.802 ± 0.007	**

Centrally nucleated and angular fibers are common characteristics of agrin cleavage-induced and age-associated sarcopenia. The percentage of soleus muscle fibers with central myonuclei was significantly increased due to neurotrypsin overexpression in female animals from line Nto1, Nto2, and Nto-moto. The increase was smaller in Nto2 than in Nto1 and could be rescued by overexpression of cleavage-resistant agrin. Aged muscles also showed an increased proportion of centrally nucleated fibers. The circularity was used as measure for the fiber shape. Decreased fiber circularity was found in muscles from Nto1, Nto2, Nto-moto, and in aged mice, indicating the appearance of more angular fibers with low circularities. Cleavage-resistant agrin but not wild type agrin restored the wild type situation. Inactive neurotrypsin or the absence of neurotrypsin had neither an influence on myonuclei position nor on fiber shape. n indicates the number of animals for each group. All fibers of each muscle were used for quantification. Data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °P < 0.05, °°P < 0.01, °°°P < 0.001 compared to Nto1.

and the loss of fibers. Another characteristic of neurotrypsin transgenic muscle was increased central nucleation of myofibers (**Table V, Fig. 16**). In wild types, the nuclei of the muscle fibers were located at the boarder of the fiber and centralized nuclei were rarely observed. Following neurotrypsin overexpression and enhanced agrin cleavage, centrally nucleated fibers appeared. Almost 3 % of the soleus muscle fibers contained centralized nuclei in transgenic animals from line Nto1. Centrally nucleated fibers are commonly recognized as regenerated myofibers[272], indicating a regeneration reaction of the muscle to the extensive fiber loss. Angular, as well as centrally nucleated fibers appeared in all mouse lines overexpressing active neurotrypsin and the extent of these alterations was consistent with the level of neurotrypsin expression and agrin cleavage (Nto1 > Nto2). The motoneuronal overexpression caused the same effect and the rescue experiment was also successful in this respect. The concomitant overexpression of cleavage-resistant agrin with neurotrypsin (Nto1/rAg) restored the wild type situation, whereas wild type agrin (Nto1/Ag) did not.

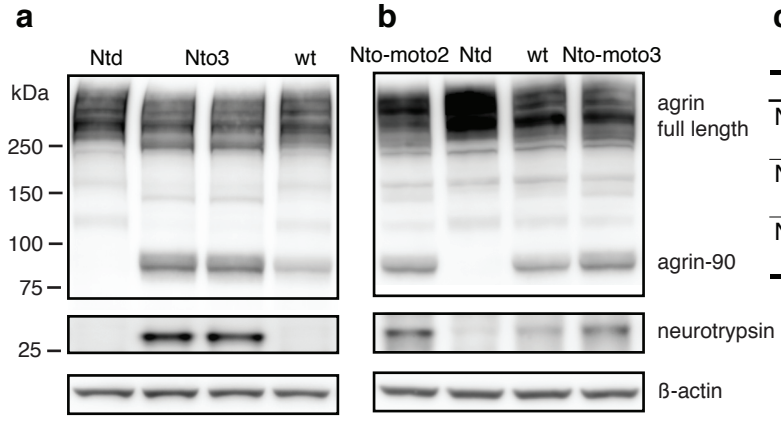
In summary, excessive cleavage of agrin, induced by neurotrypsin overexpression in motoneurons, was responsible for the degenerative muscular alterations in young adult mice. Concomitant overexpression of neurotrypsin and a cleavage-resistant agrin variant completely restored the wild-type situation in all aspects tested. Denervation of the individual muscle fibers is suspected to induce atrophy and degeneration of a subset of muscle fibers. Muscle intrinsic regeneration mechanisms are activated but fail to prevent the massive loss of skeletal muscle fibers. However, some of the remaining fibers become hypertrophied to compensate, at least partially, the reduction in muscle thickness following fiber loss.

Similar alterations of the muscle morphology have previously been described in senescence as a consequence of the age-associated muscle degeneration referred to as sarcopenia. One of the most striking characteristics of sarcopenia is the loss of muscle fibers. Unlike other catabolic conditions such as cachexia or disuse that lead to muscle atrophy, sarcopenia is the result of a loss of muscle

fibers, as well as a marked heterogeneity in the CSA of the remaining myofibers[232,238]. Skeletal muscles of aged mice were analyzed for comparison of the neurotrypsin- and aging-induced effects (**Fig. 15, 16f**). Indeed, strikingly similar muscle morphological changes were found. As in conditions with enhanced agrin cleavage in young adult mice, loss of myofibers and increased fiber size variability were observed with aging (**Tables II, III, Fig. 17**). Compensatory fiber hypertrophy resulted in increased mean fiber thickness (**Table IV**). Angular fibers and centralized nuclei indicated ongoing denervation and muscle fiber regeneration, respectively (**Table V**). A significant reduction in whole muscle CSA was not found but the same tendency was observed (**Table IV**). The gain in body mass in aged animals that increases the loading of the muscle may induce the compensatory mechanism to a higher extend than in transgenic animals. Interestingly, the EDL muscle showed significant age-associated reduction in mass, whereas the soleus muscle mass did not change with age, despite a similar drop in muscle fiber number (**Table I**).
In summary, surprisingly similar morphological changes were found in the muscles of young adult mice exhibiting enhanced agrin cleavage and in aged wild type mice. This finding suggested a common etiology for both, agrin cleavage- and aging-induced muscle degeneration. Excessive agrin cleavage seems to precociously induce sarcopenia in young adult mice.

3.4 Additional transgenic mouse lines exhibit the same phenotypes

Beside the Nto1, Nto2, and Nto-moto additional mouse lines were investigated, including a third line overexpressing neurotrypsin neuronally (Nto3), and two lines overexpressing neurotrypsin motoneuronally (Nto-moto2 and Nto-moto3). Western blotting confirmed the overexpression of neurotrypsin and the accumulation of agrin-90 in line Nto3, Nto-moto2 and Nto-moto3 (**Fig. 19a, b**). The number of fibers of the soleus was assessed to get an impression about the severity of the muscle wasting effect (**Fig. 19c**). All of the transgenic lines exhibited a decreased number of fibers, confirming the previous findings. Loss of muscle fibers is a reliable and repeatable result of motoneuronal neurotrypsin overexpression. Line specific effects, such as insertion place or copy number of the transgene, may influence the expression level of neurotrypsin and hence, the severity of muscle fiber loss.



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	genotype	n	number of fibers
Nto3	wt	9	937 ± 48
	Nt	15	643 ± 38 ***
Nto-moto2	wt	5	941 ± 44
	Nt/cre	4	671 ± 97 *
Nto-moto3	wt	6	911 ± 19
	Nt/cre	3	791 ± 16 *

Figure 19 Neurotrypsin overexpression is associated with enhanced agrin cleavage and skeletal muscle fiber loss in additional mouse lines. Western blots from spinal cord homogenates from P10 mice overexpressing neurotrypsin neuronally (**a**) or motoneuronally (**b**). All transgenic mice showed an increased immunoreactivity for neurotrypsin and for agrin-90. ß-actin was used as loading control. (**c**) The number of fibers is significantly reduced in transgenic animals from line Nto3, Nto-moto2, and Nto-moto3 compared to wild type littermates. n indicates the number of animals. Data represent mean ± s.d. *P < 0.05, ***P < 0.001.

3.5 Influence of enhanced agrin cleavage on postnatal skeletal muscle development

The postnatal development of skeletal muscles of neurotrypsin transgenic animals was analyzed to assess the time scale of agrin cleavage-induced skeletal muscle degeneration. A temporal correlation between onset of neurotrypsin overexpression and skeletal muscle fiber loss was expected. The Thy 1 expression cassette was reported to drive transgene expression with a relatively late onset around postnatal days 6-10[634]. However, previous findings from our lab indicated that neurotrypsin overexpression from the Thy 1 promotor may start slightly earlier (Bolliger et al., in preparation). Soleus muscles from mice at different ages between 10 days and 10 months were collected and the number of fibers was counted on cross-sections. A mouse line overexpressing neurotrypsin motoneuronally (Nto-moto2) was used. At P10 wild type and transgenic animals had a similar number of myofibers (**Fig. 20a**). Shortly after, the number of fibers started to decrease in transgenic animals. In wild types, however, the fiber number increased with age in the first two

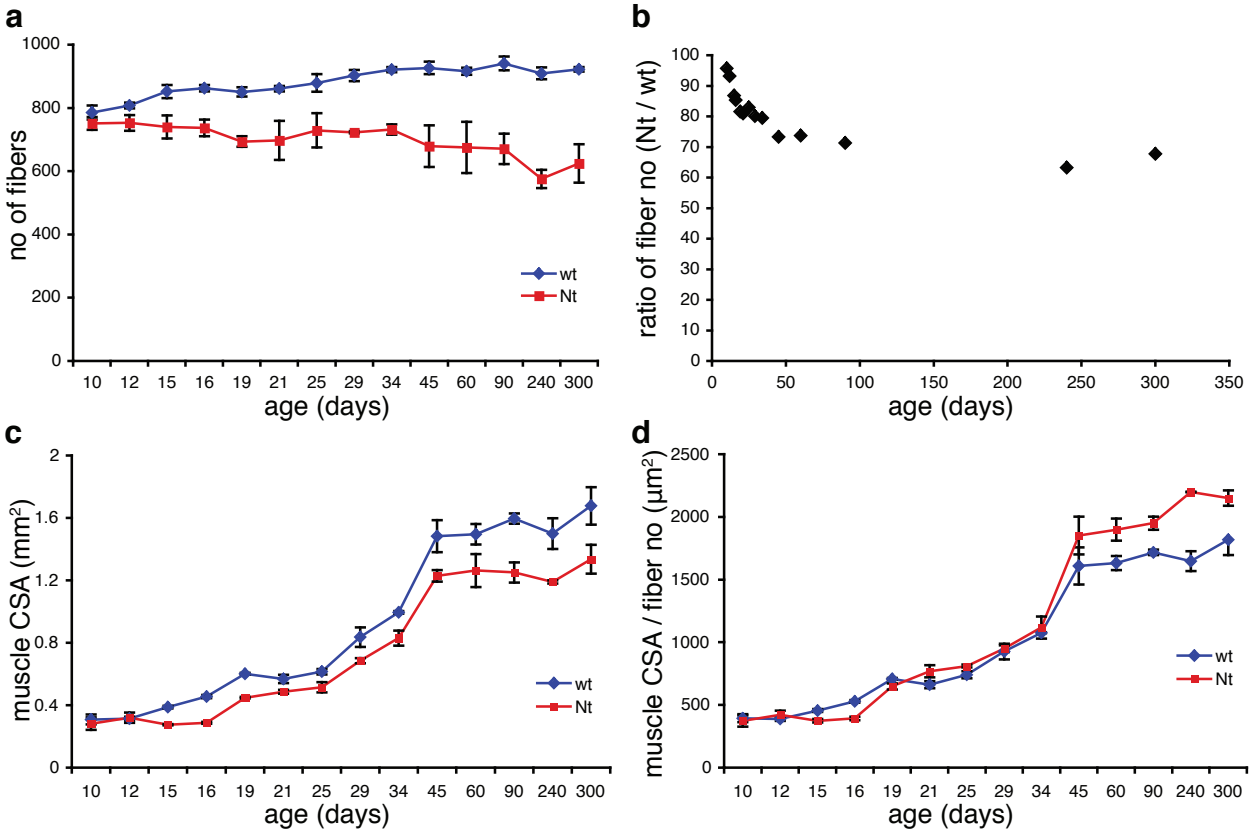


Figure 20 The sarcopenic phenotype is installed in a couple of weeks. (**a**) Developmental study of the soleus muscle fiber number in animals from line Nto-moto2 starting 10 days postnatally (P10). In wild type mice (wt), growth of the muscle was associated with a slight increase in the number of fibers during the first two to three weeks after birth, whereas in transgenic animals muscle fibers were lost in this time period. (**b**) The ratio of the fiber numbers of transgenic relative to wt muscles greatly decreased in the first 50 days after birth and remained unchanged thereafter. (**c**) The soleus muscle CSA increased in the first month postnatally, attributable to the growth of the muscle. Differences between neurotrypsin-overexpressing and wt animals mostly parallel the findings from the fiber numbers. At P10 and P12, soleus muscle thickness was the same in transgenic and wt animals. A difference in muscle CSA was observed at P15 and increased until about day 50. Thereafter, muscle thickness remained constant in both transgenic and wt animals. (**d**) Mean muscle fiber thickness (estimated from the division of muscle CSA by fiber number) did not differ between wt and transgenic animals during postnatal development. After day 50, the individual myofibers were, in average, thicker in transgenic than in wt animals, most likely due to compensatory hypertrophy. This difference remained constant during adulthood. n ≥ 2 animals per genotype and time point. Error bars represent s.d.

to three weeks postnatally. At P15 a clear difference was visible, which was further increased until about postnatal day 60 (**Fig. 20b**). Later, the ratio of fiber numbers stayed constant. The start of the muscle wasting, around 10 days postnatally, appeared to be delayed in regard to the onset of neurotrypsin overexpression, which was suspected to start around five days postnatally. Nevertheless, it is plausible that the onset of overexpression, the cleavage of agrin, as well as atrophy and degeneration of the muscle fibers need some time before the impact on the muscle morphology can be detected. After P60, an age when the mice are thought to be mature, the number of fibers stayed remarkably constant. During initial postnatal growth of the muscle, in contrast, the action of neurotrypsin appears to be especially fatal, installing the basic muscle

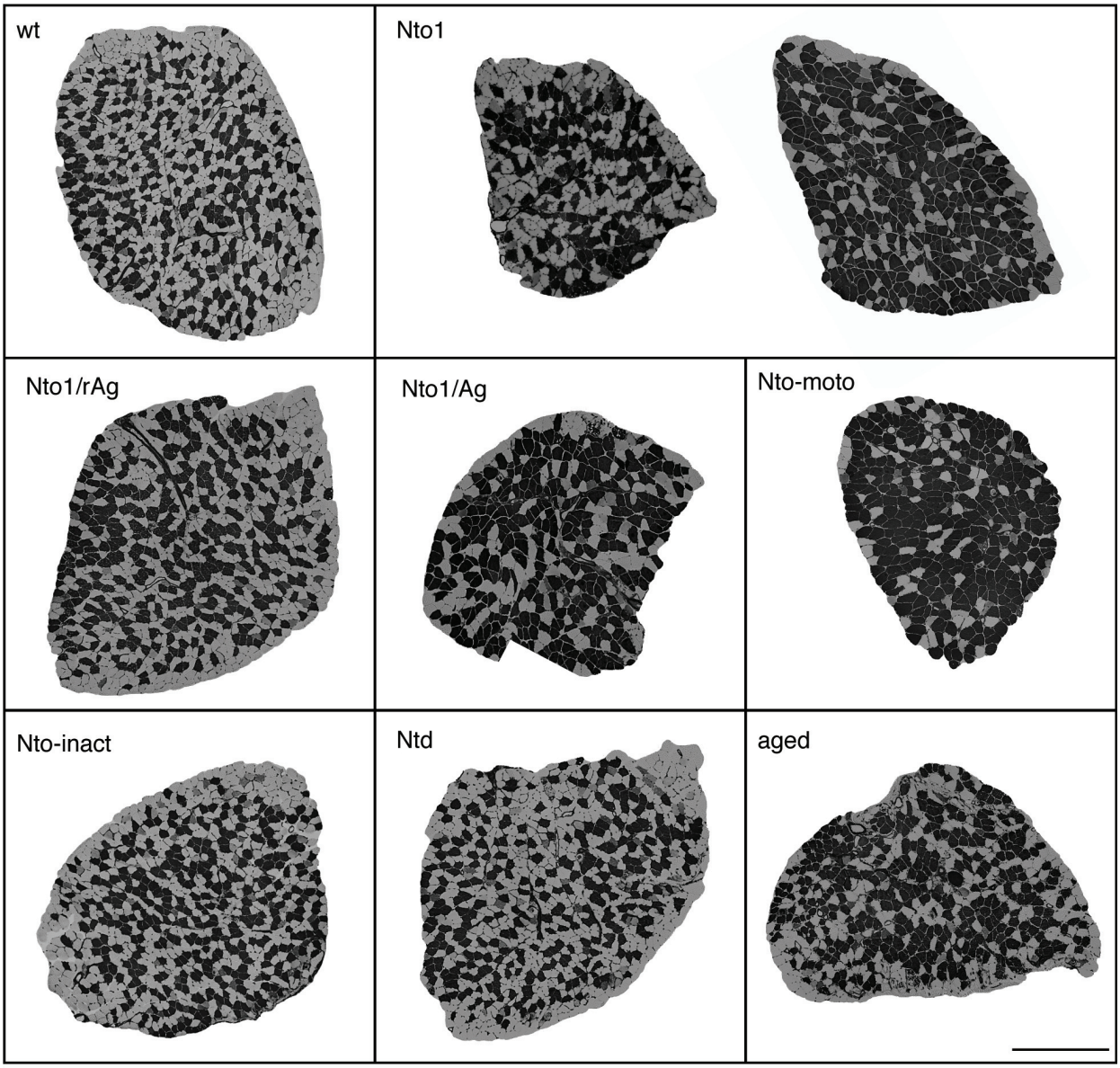
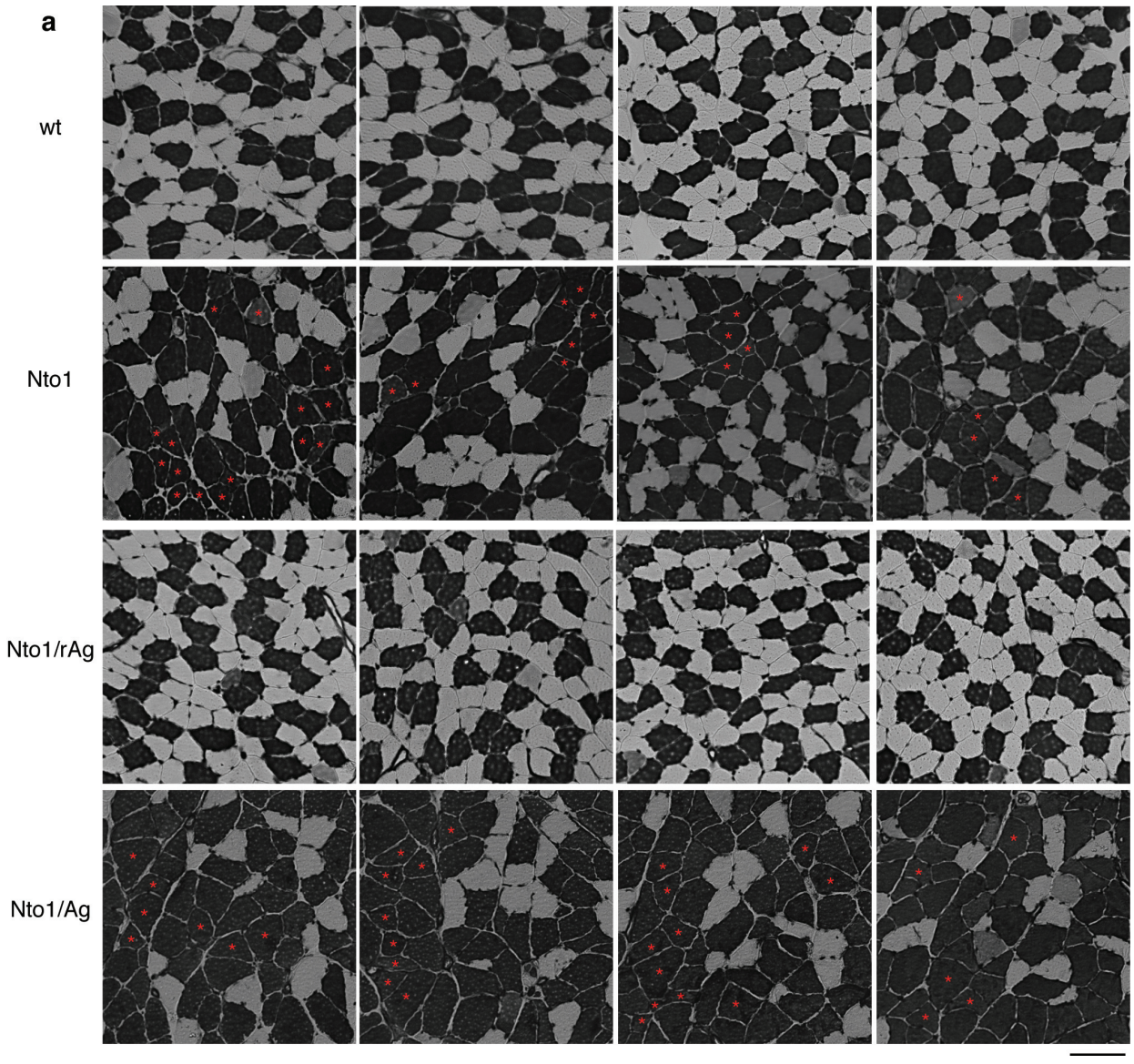


Figure 21 Relative increase in type I fibers and type grouping upon neurotrypsin overexpression and aging. Soleus muscle cross sections from female animals, stained for myofibrillar actomyosin ATPase (mATPase) activity after preincubation at pH 4.3. Only the slow, type I fibers are stained. Muscles from Nto1, Nto-moto, and aged animals showed a relative increase in darkly stained type I fibers compared to adult wild type animals. In addition, fiber type grouping was observed. Two cross-sections from Nto1 animals are shown to demonstrate the substantial variation in the proportion of the fiber types among neurotrypsin-overexpressing animals. The overexpression of cleavage-resistant agrin together with neurotrypsin restored the wild type situation, whereas wild type agrin did not. Inactive neurotrypsin and the absence of neurotrypsin did not affect the fiber type distribution. Scale bar, 0.5 mm.

phenotype in a couple of days.
Muscle CSA was increased during the first 6 weeks postnatally, regardless whether neurotrypsin was overexpressed or not (**Fig. 20c**). Differences in muscle thickness between transgenic animals and wild type littermates were observed at day 15 postnatally, at the time when also the number of muscle fibers started to differ. During adulthood, muscle CSA and fiber number did not change greatly neither in wild type nor in transgenic animals. Therefore, the difference in muscle thickness was most likely a consequence of the muscle fiber loss. Growth of the surviving individual muscle fibers appeared not to be affected by neurotrypsin overexpression, since mean fiber CSA was similar in transgenic and wild type mice during postnatal development (**Fig. 20d**). At day 45, however, mean fiber thickness started to differ in transgenic compared to wild type animals, suggesting compensatory hypertrophy as a reaction to the muscle fiber loss.

3.6 Loss of type II fibers exceeds loss of type I fibers both in young adult mice with enhanced agrin cleavage and in aged wild type mice

Muscle type specific actomyosin ATPase (mATPase) staining was used to analyze the soleus muscle for the occurrence of slow (type I) and fast (type II) muscle fibers. Type I fibers are



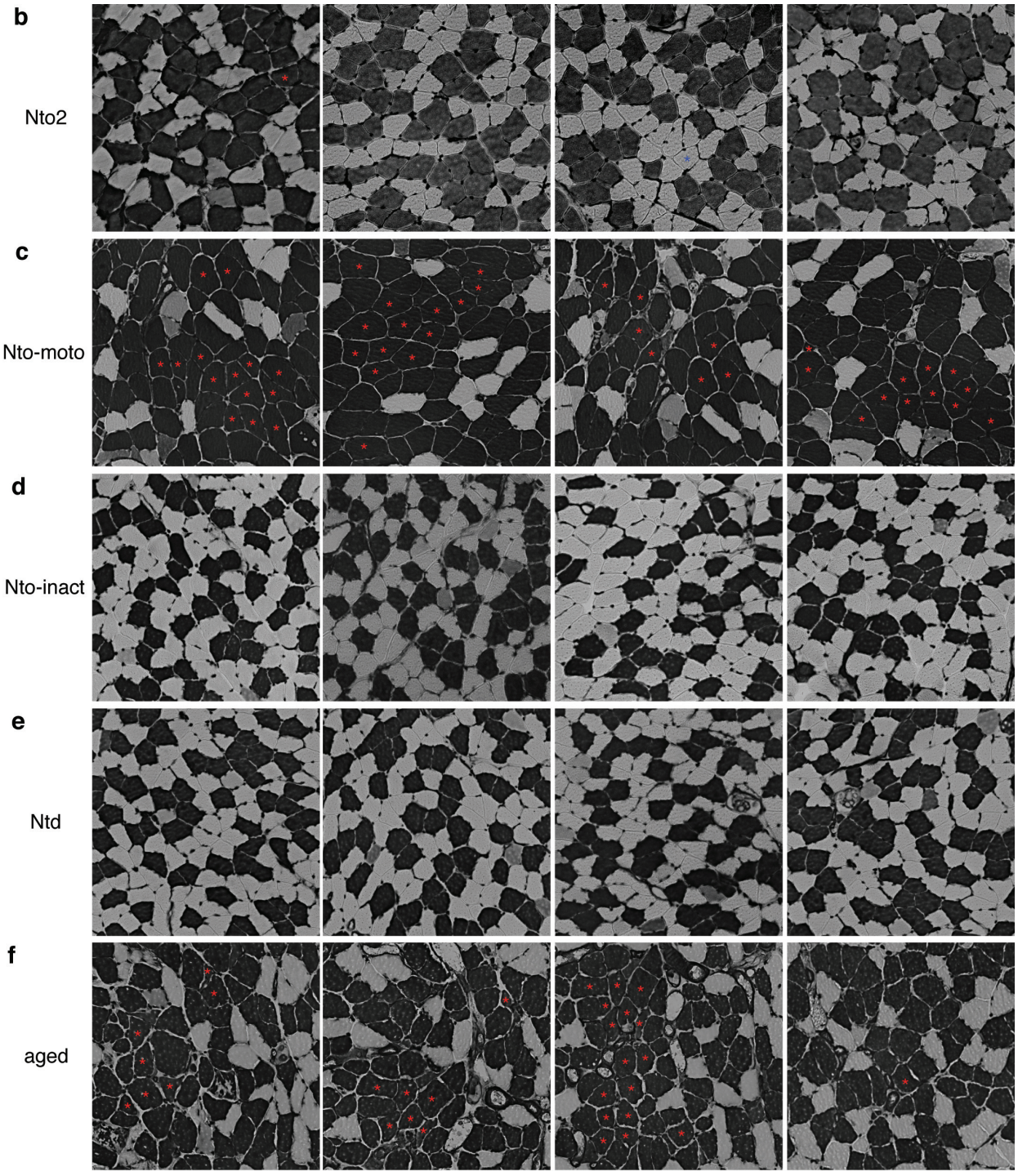


Figure 22 Type I fiber preference and type grouping are common features of neurotrypsin transgenic and aged muscles. Soleus muscle cross-sections from female animals stained for mATPase activity after preincubation at pH 4.3. Only the slow, type I fibers are stained. (**a**) Comparison of cross-sections from wt, Nto1, Nto1/rAg and Nto1/Ag animals. Increased proportion of type I fibers and accumulation of enclosed fibers (asterisks) were observed in Nto1 and Nto1/Ag animals. The accumulation of enclosed fibers indicated fiber type grouping. Concomitant overexpression of neurotrypsin-resistant agrin and neurotrypsin restored the wild type situation. (**b**) Cross-sections from Nto2 mice exhibited only minor effects. (**c**) Motoneuronal neurotrypsin overexpression was sufficient to induce both relative increase in type I muscle fibers and accumulation of enclosed fibers. (**d, e**) Cross-sections from mice overexpressing inactive neurotrypsin (Nto-inact) and from neurotrypsin deficient mice (Ntd) were not distinguishable from wild type cross-sections. (**f**) Type I fiber preference and type grouping was also observed in senescence. Scale bars, 0.1mm.

known to express an acid-stable myosin isoform, whereas type II fibers express an acid-labile myosin isoform. Preincubation at pH 4.3 was used to abolish myosin activity in type II fibers and subsequent staining for mATPase activity made it possible to discriminate between darkly stained type I fibers and unstained type II fibers. The number and proportion of type I fibers was determined. The proportion of type I fibers was higher in female (about 40 %) than in male (about 30 %) wild type mice. However, the comparison between wild type and transgenic animals revealed striking differences for both males and females.

In females, a significant increase in the proportion of type I fibers was observed following neurotrypsin overexpression (**Fig. 21, 22, 23a**). However, absolute numbers were decreased for both type I and type II fibers (**Table VI**). The severity of the effect was in good correlation with the agrin cleavage (Nto1 > Nto2) and the overexpressing of neurotrypsin selectively in motoneurons (Nto-moto) also induced the preference towards type I fibers. Inactive neurotrypsin or the absence of neurotrypsin did not cause any effect. The additional overexpression of resistant agrin restored the wild type situation, whereas additional overexpression of wild type agrin did not. Thus, neurotrypsin-dependent cleavage of agrin at its α and β sites induced the preference towards type I fibers. Interestingly, the concomitant overexpression of a cleavable agrin variant and neurotrypsin resulted in a more pronounced type I fiber preference than the overexpression of neurotrypsin alone (**Fig. 23a**). The number of type I fibers was significantly increased in the

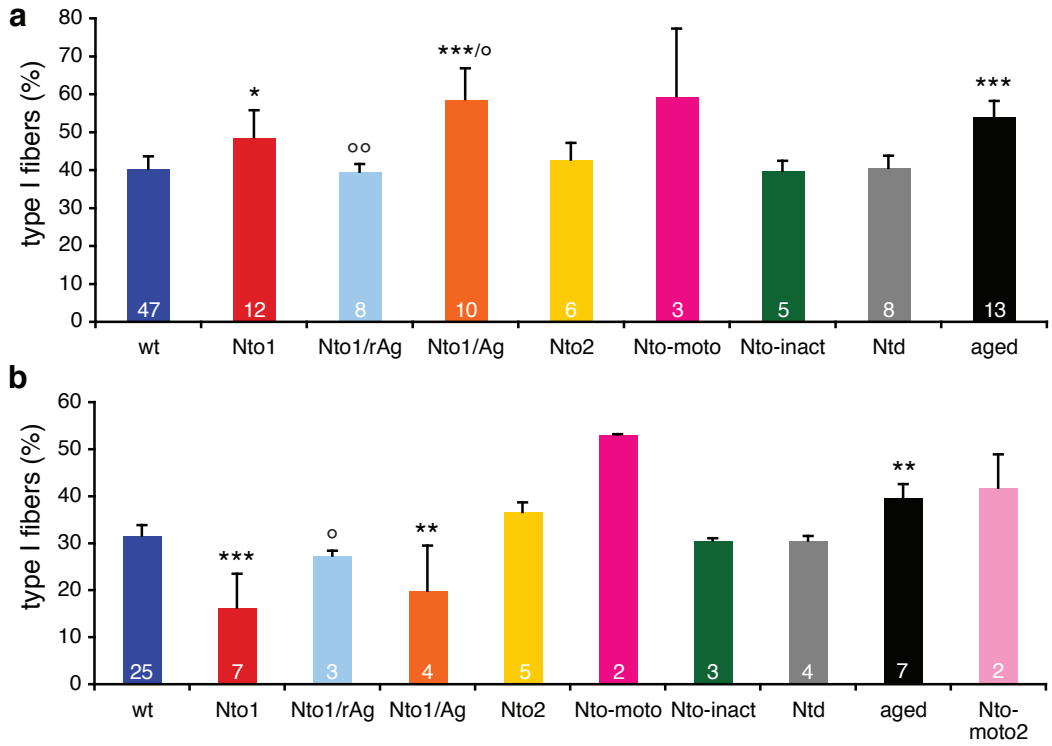


Figure 23 Quantification of the proportion of type I fibers in the soleus muscle of female (**a**) and male (**b**) mice. (**a**) Note the relative increase in type I fibers due to overexpression of active neurotrypsin in line Nto1 and Nto-moto, and due to aging. Line Nto2 showed only a minor effect. Concomitant overexpression of cleavage-resistant agrin and neurotrypsin restored the wild type situation (Nto1/rAg). Interestingly, the overexpression of wild type agrin together with neurotrypsin (Nto1/Ag) further increased the proportion of type I fibers compared to the overexpression of neurotrypsin alone (Nto1). (**b**) In males, type I fiber preference was observed in muscles from Nto2, Nto-moto and for aged mice. In line Nto1, the proportion of type I fibers was decreased. This effect could be rescued by additionally overexpressing cleavage-resistant agrin.

The number of animals for each group is indicated inside each bar. Error bars represent s.d. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °P < 0.05, °°P < 0.01, °°°P < 0.001 compared to Nto1.

Table VI
Relative and absolute number of type I fibers in female soleus muscle

line	genotype	n	type I fibers (%)	no of type I fibers
Nto1	wt	17	40 ± 4	378 ± 49
	Nt	12	49 ± 7 *	313 ± 54 **
x rAg	Nt/rAg	8	40 ± 2 °°	371 ± 25 °
x Ag	Nt/Ag	10	59 ± 8 ***/°	372 ± 48 °
Nto2	wt	4	41 ± 2	382 ± 20
	Nt	6	43 ± 4	368 ± 34
Nto-moto	wt	6	42 ± 3	402 ± 23
	Nt/cre	3	60 ± 18	349 ± 7 *
Nto-inact	wt	5	39 ± 2	362 ± 10
	iNt	5	40 ± 3	377 ± 40
Ntd	wt	4	41 ± 3	380 ± 36
	ko	8	41 ± 3	381 ± 23
	adult	11	39 ± 3	377 ± 28
	aged	13	54 ± 4 ***	437 ± 34 **

The absolute number of type I fibers was decreased in line Nto1 and Nto-moto, despite type I fiber preference. Resistant agrin rescued this effect. The pronounced type I fiber preference in Nto1/Ag animals even prevented absolute loss of type I fibers. In aged muscles, the absolute number of type I fibers was actually increased. n indicates the number of animals for each group. Data represent mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °P < 0.05, °°P < 0.01.

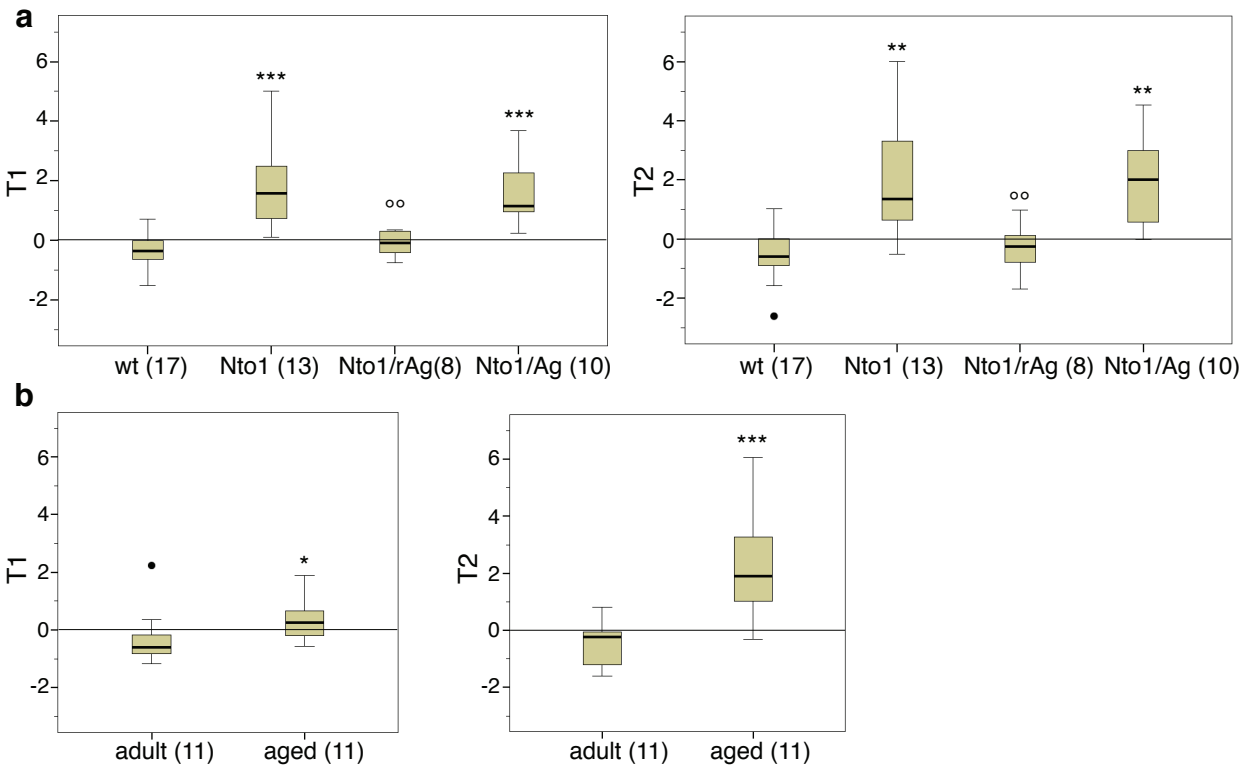
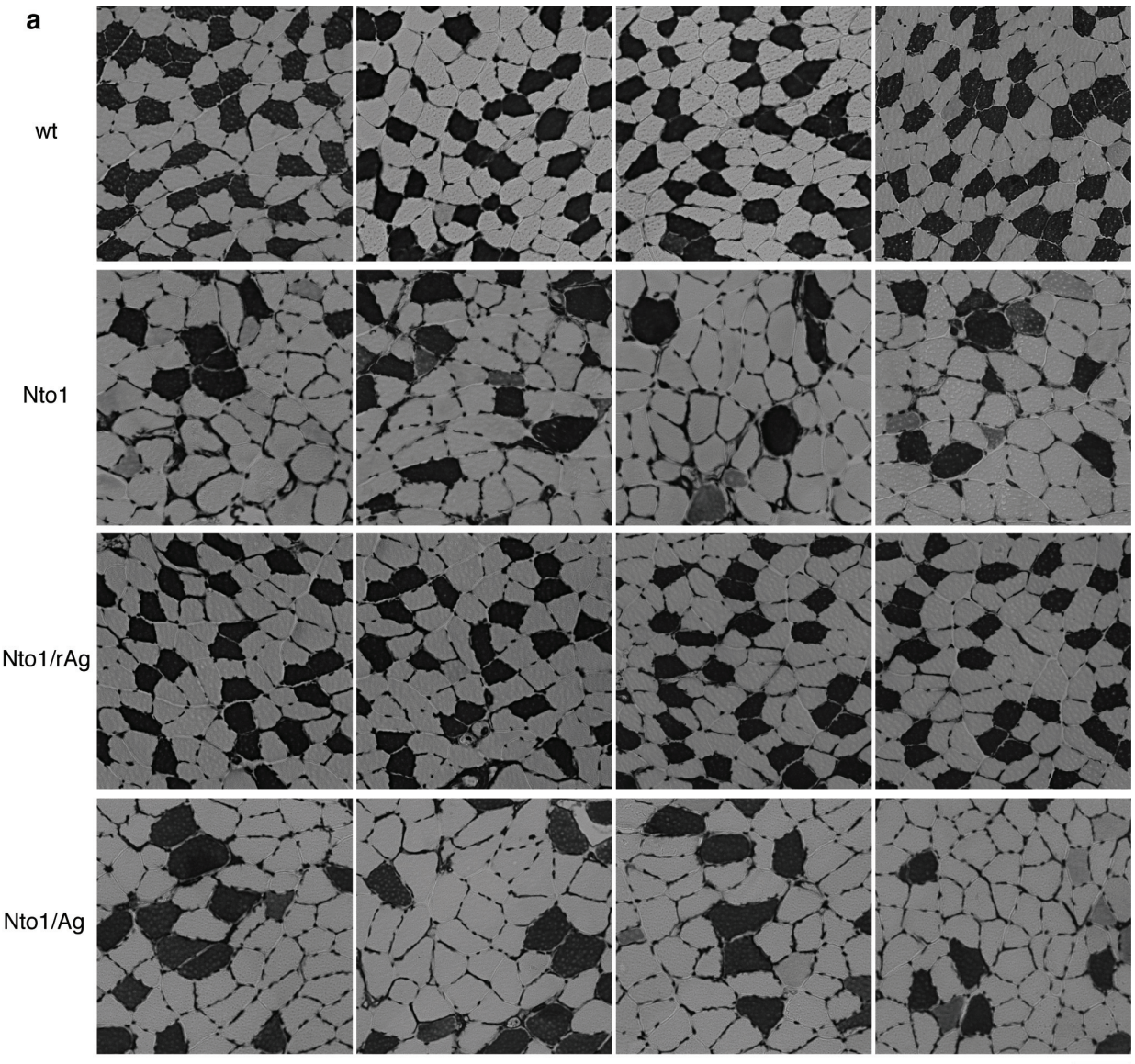


Figure 24 Enhanced agrin cleavage and aging induce fiber type grouping. Fiber type grouping in female soleus muscles was quantified using the method from Lexell et al.[267]. T values equal 0 indicate a random fiber type arrangement, T values lower than 0 fiber type segregation, and T values above 0 fiber type grouping. T1 and T2 values represent arrangement of type I and type II fibers, respectively. (**a**) In adult wild type muscles T1 and T2 were slightly negative. Following neurotrypsin overexpression T1 and T2 were increased to more than 1, indicating grouping of both type I and type II fibers. Concomitant overexpression of cleavage-resistant agrin and neurotrypsin restored the wild type situation, whereas wild type agrin did not. (**b**) Positive T values in aged mice indicated age-associated fiber type grouping. Note that the age-dependent increase in T2 was clearly stronger than the increase in T1.
The number of animals for each group is indicated in parentheses. The top of the box represents the upper quartile, the bottom the lower quartile, and the black line the median. The whiskers indicate the highest and lowest values that are no outliers. Filled circles represent outliers that do not lay within 1.5 fold the interquartile range. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °P < 0.05, °°P < 0.01, °°°P < 0.001 compared to Nto1.

double transgenic animals compared to single transgenic littermates, although the overall number of fibers was the same. Thus, transgenic wild type agrin influences the fiber type distribution in neurotrypsin-overexpressing animals without actually affecting the fiber number. The type I fiber preference in Nto1/Ag animals was so extensive that the absolute number of type I fibers stayed at wild type levels, despite the 30% drop in fiber number (**Table VI**).

The increase in the proportion of type I fibers in concert with a reduction of both type I and type II fibers in neurotrypsin transgenic animals could be the result of two processes: The predominant loss of type II fibers, or the takeover of type II fibers by adjacent type I motoneurons, promoting a fiber type change. The later happens after denervation and reinnervation of muscle fibers and is usually accompanied by fiber type grouping. The method of enclosed fibers, developed by Lexell and Downham[267] was used to quantify fiber type grouping. This method depends on T1 and T2 values, which are measures for the randomness of the arrangement of type I and type II fibers, respectively. T values equal to 0 indicate a random distribution, T values lower than 0 indicate fiber type segregation, and T values above 0 indicate fiber type grouping. Both T1 and T2 value was significantly increased in conditions with enhanced agrin cleavage, suggesting fiber type grouping (**Fig. 24a**). Accordingly, clusters of histochemically identical fibers were observed in neurotrypsin-overexpressing muscles (**Fig. 21, 22**). Concomitant overexpression of cleavage-



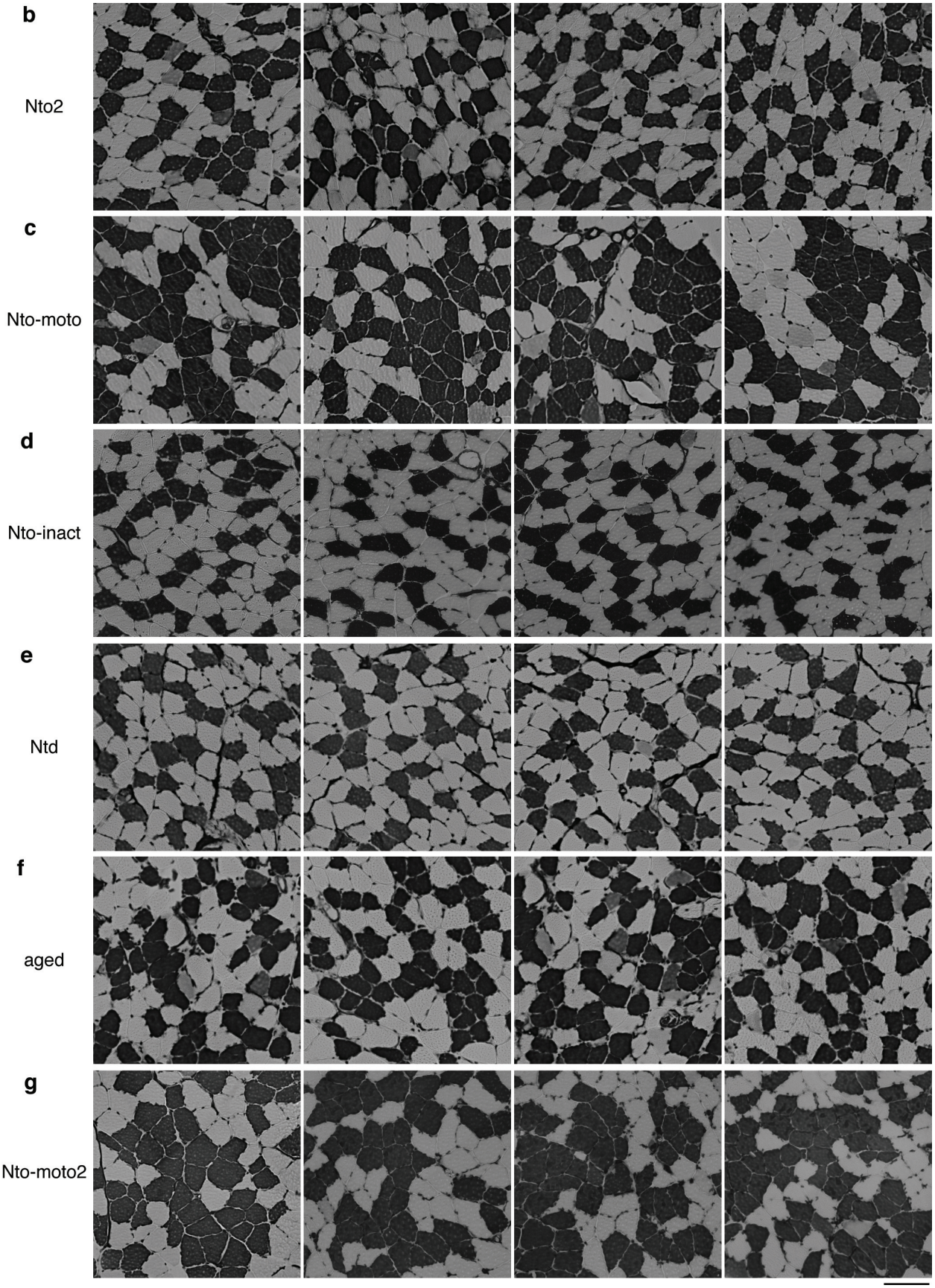


Figure 25 Neurotrypsin overexpression does not cause a consistent increase in type I fibers in male muscles. Soleus muscle cross-sections from male animals stained for mATPase activity after preincubation at pH 4.3. Only the slow, type I fibers are stained. (**a**) Neurotrypsin overexpression caused a decrease in the proportion of type I fibers in

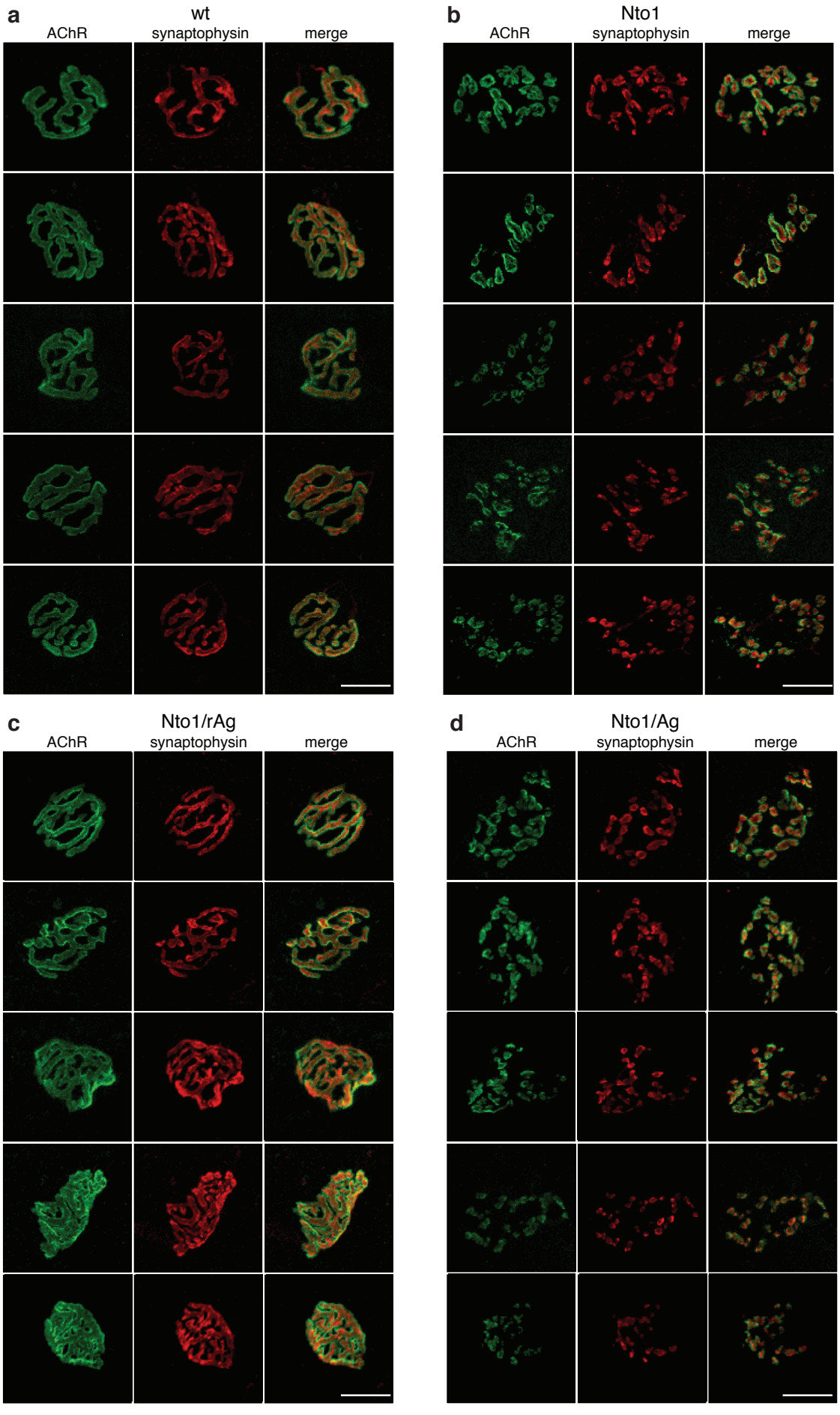
line Nto1. Cleavage resistant agrin rescued from this phenotype, wild type agrin did not. (**b**) Cross-sections from line Nto2 showed a small increase in type I fiber proportion. (**c**) Type I fiber preference and an accumulation of enclosed fiber was observed in soleus muscles from Nto-moto animals. (**d**, **e**) Overexpression of inactive neurotrypsin and the absence of neurotrypsin did not affect the muscle morphology. (**f**) Aging was accompanied by a relative increase in type I fibers and a tendency for fiber type grouping. (**g**) Muscle cross-sections from a second mouse line overexpressing neurotrypsin motoneuronally exhibited type I fiber preference and accumulation of enclosed fiber. Scale bars, 0.1 mm.

resistant agrin and neurotrypsin rescued from the grouping phenotype, whereas concomitant overexpression of wild type agrin and neurotrypsin did not. Enhanced agrin cleavage induced fiber type grouping in young adult mice, indicating continuous denervation and reinnervation with consecutive type change. Whether this type change is responsible for type I fiber preference can't be deduced, since a grouping of both type I and type II fibers was observed. Thus, reinnervation by both type I and type II motoneurons had to take place. Nevertheless, the ratio of reinnervation could be responsible for the increase in type I fiber proportion.
In males, type I fiber preference and type grouping were also detected in soleus muscles of Nto2 and Nto-moto animals (**Fig. 23b, 25b, c**). In line Nto1, however, the situation was completely different. A significant decrease in the proportion of type I fibers was observed in transgenic mice compared to wild type littermates (**Fig 23b, 25a**). In severe cases, only few type I fibers were left. The rescue experiment, using transgenic expression of resistant agrin, was successful, indicating that the type II preference was caused by the cleavage of agrin and not by an unspecific side effect, such as the disruption of a gene due to the transgenic insertion. However, it is difficult to explain the differences between males and females from the line Nto1, especially because both males and females behaved similarly in all other lines. An additional mouse line was analyzed to clarify the situation. The proportion of type I fibers in male and female mice from line Nto-moto2 was clearly increased upon neurotrypsin overexpression, confirming the findings from line Nto2 and Nto-moto (**Fig. 23b, 25g**). The contrary effect in males from line Nto1 appeared to be an exception that was not observed in another neurotrypsin-overexpressing condition.
Again, aging was accompanied by remarkable similar effects. The proportion of type I fiber was significantly increased in the soleus muscle of aged compared to adult animals and fiber type grouping was observed, very much resembling the situation in neurotrypsin transgenic mice (**Fig. 21, 22f, 23, 25f**). However, type I fiber preference was more pronounced in aged than in Nto1 animals, although the phenotype was milder in regard to other histological parameters (**Fig. 23**). Therefore, the absolute number of type I fibers was actually increased in aged compared to adult mice, despite the total loss of muscle fibers (**Table VI**). Nevertheless, like in the transgenic situation, denervation and reinnervation processes are likely to have taken place, since a clear type grouping was observed (**Fig. 24b**). For aged animals, T2 values were significantly greater than T1 values, suggesting preferential type II fiber grouping. However, several assumptions have to be taken for statistical assessment of type grouping and the differences in T1 and T2 values may not necessarily reflect a biological process but could also have methodological reasons.

3.7 Deterioration of the NMJ is responsible for age- and agrin cleavage-induced sarcopenia

3.7.1 Excessive agrin cleavage and aging are associated with fragmentation of the NMJ

The connection between motoneuronal neurotrypsin overexpression and muscle fiber degeneration was though to be at the NMJ. Neurotrypsin overexpression in the motoneuron induces enhanced agrin cleavage at the NMJ. This process could influence the structure of the NMJ, because agrin


a
wt
AChR
synaptophysin
merge
b
Nto1
AChR
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merge
c
Nto1/rAg
AChR
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d
Nto1/Ag
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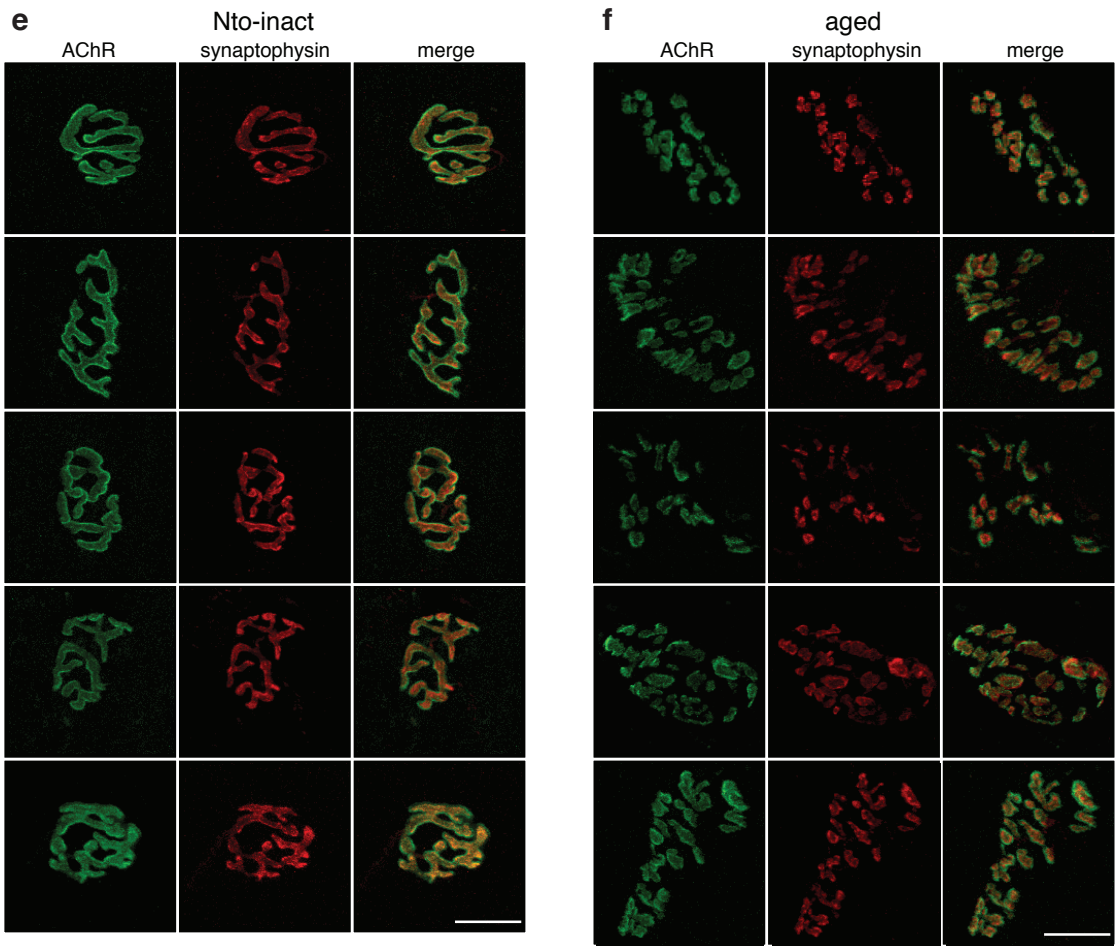


Figure 26 Highly fragmented NMJs on the soleus muscles of neurotrypsin-overexpressing and aged mice. The NMJs were visualized by fluorescent staining of synaptophysin (red) to label the presynaptic nerve ending and acetylcholine receptors (green) to label the postsynaptic muscle membrane. Antibodies against synaptophysin followed by Cy3-conjugated secondary antibodies, and Alexa-488 conjugated α-bungarotoxin were applied. Pictures were taken with a Leica LCS SP2 confocal microscope using a 63x objective. (**a**) NMJs on wild type muscles exhibited the typical pretzel-like appearance. (**b**) Highly fragmented NMJs appeared upon neurotrypsin overexpression. Pre-and postsynaptic elements were still perfectly aligned in the majority of cases. (**c**) Additional overexpression of a cleavage-resistant agrin variant restored the pretzel shapes. NMJs on Nto1/rAg double transgenic animals often appeared very compact, with relatively small receptor-free areas (bottom rows). (**d**) The overexpression of wild type agrin did not rescue from the fragmentation phenotype. (**e**) The overexpression of a catalytically inactive neurotrypsin did not have any influence on NMJ structure. (**f**) Fragmented NMJs were also observed on the soleus muscle of aged mice. Scale bars, 20 μm.

cleavage has been shown to regulate the stability of the NMJ (Bolliger et al., in preparation).
We used the soleus muscle of female mice to study the effect of neurotrypsin overexpression on the NMJ. The synaptic terminals were visualized by indirect immunofluorescence with antibodies against synaptophysin and the postsynaptic specialization by staining the AChRs with fluorophore-conjugated α-bungarotoxin. Normally, adult NMJ show a typical pretzel-like appearance, with a perfect alignment of the pre- and postsynaptic elements. The NMJs of neurotrypsin-overexpressing mice exhibited enhanced fragmentation of both pre- and postsynaptic sites (**Fig. 26a, b**). The pretzel-like structure was replaced by numerous small spots but the alignment of pre- and postsynaptic sites was still maintained in the majority of NMJs. The degeneration of the NMJ was quantitatively analyzed by counting the number of fragments per junction and by measuring the area, which was occupied by pre- and postsynaptic elements (**Fig. 27, 28**). In wild type animals, most of the presynaptic terminals (about two third) were composed of one to four

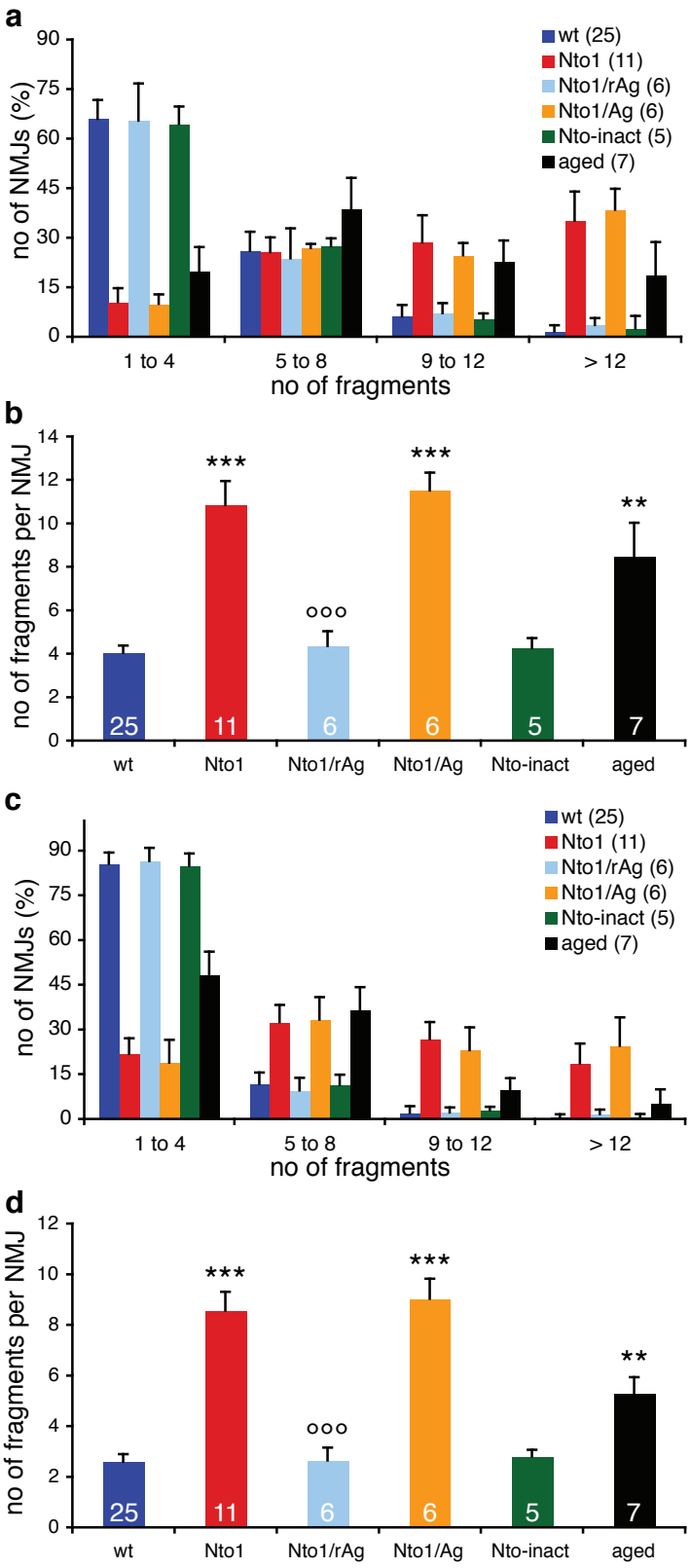


Figure 27 Quantification of NMJ fragmentation on the soleus muscles of Nto1, Nto-inact and aged mice. ImageJ was used to determine the number of fragments per endplate on maximal projections of confocal stacks from presynaptic synaptophysin (**a**, **b**) and postsynaptic α-bungarotoxin (**c**, **d**) stainings. (**a**, **c**) The proportions of highly fragmented pre- and postsynapses (more than 12 fragments) were increased in mice overexpressing active neurotrypsin. Additional overexpression of resistant agrin rescued from the fragmentation (Nto1/rAg), whereas wild type agrin did not (Nto1/Ag). Inactive neurotrypsin did not induce NMJ fragmentation. The fragmentation pattern in aged animals was strikingly similar. (**b**, **d**) The averaged number of fragments per NMJ was significantly increased in transgenic animals from line Nto1 and in aged animals. Cleavage-resistant agrin restored the wild type situation, cleavable agrin did not. Note that the number of postsynaptic fragments was always lower than the number of presynaptic fragments, due to slightly different staining patterns. Postsynaptic signals usually were more widespread than presynaptic signals. The number of animals for each group is indicated in parentheses (**a**, **c**) and inside each bar (**b**, **d**), respectively. All available endplates (at least 30 per animal) were quantified. Error bars represent s.d. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to wild type littermates or adult controls, $^{°°°}P < 0.001$ compared to Nto1.

fragments, whereas highly fragmented terminals with more than 12 fragments were rare. In neurotrypsin-overexpressing animals, the amount of normal, pretzel-like presynapses was dramatically decreased (to about 10 % in line Nto1) and the number of highly fragmented presynapses was significantly increased (**Fig. 27a**). About one third of the transgenic nerve endings were composed of more than 12 fragments. Consequentially, the averaged number of fragments per endplate was increased (**Fig. 27b**). Furthermore, the presynaptic area was significantly reduced in neurotrypsin transgenic animals (**Fig. 28a**). Thus, fragmentation does not only reflect a dispersal of existing presynaptic sites but also a loss of synaptic territory. Similar results were obtained for the postsynaptic site (**Fig. 27c, d, 28b**). The mouse line overexpressing inactive neurotrypsin did not show an increased proportion of highly fragmented NMJs or a loss of synaptic area, confirming the crucial role

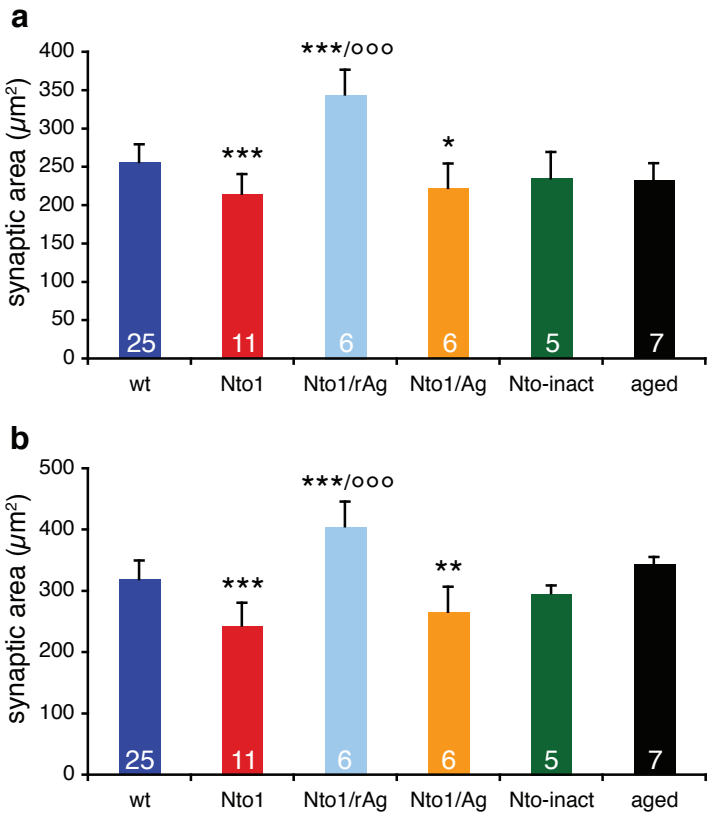


Figure 28 Loss of synaptic area following neurotrypsin overexpression. The fluorescent area was measured after application of antibodies against synaptophysin (**a**) and α-bungarotoxin (**b**) to label pre- and post-synaptic sites, respectively. Both pre- and postsynaptic areas were significantly reduced in neurotrypsin-overexpressing animals compared with wild type littermates. Concomitant overexpression of resistant agrin together with neurotrypsin not only restored the wild type situation, it actually resulted in an increase in synaptic area. Wild type agrin had no rescue effect. The overexpression of inactive neurotrypsin and aging did neither influence pre- nor postsynaptic area. Note that postsynaptic area was higher than presynaptic area, since postsynaptic signals were more widespread. The number of animals for each group is indicated inside each bar. All available endplates (at least 30 per animal) were quantified. Error bars represent s.d. *P < 0.05, **P < 0.01, ***P < 0.001 compared to wild type littermates or adult controls, °°°P < 0.001 compared to Nto1.

of the proteolytic activity of neurotrypsin (**Fig. 26e, 27, 28**). Concomitant overexpression of a cleavage-resistant agrin variant and neurotrypsin rescued from the fragmentation phenotype, restoring the typical pretzel-like appearances of the NMJs (**Fig. 26c, 27**). The synaptic area in the Nto1/rAg double transgenic animals was actually increased compared to wild-type littermates (**Fig. 28**). Interestingly, endplates with more compact shapes, represented by a smaller proportion of receptor-free area, were frequently observed in these animals and mainly contributed to the increase in synaptic area (**Fig. 26c**). Overexpression of a wild-type agrin variant together with neurotrypsin did not show any rescue effect (**Fig. 26d, 27, 28**).

The pathogenic mechanisms resulting in age-associated and neurotrypsin-induced muscle wasting were thought to be similar, due to the striking resemblance in regard to movement, strength and muscle morphology. To substantiate this hypothesis, the NMJs of aged mice were analyzed. Indeed, structural alterations that were strikingly similar to those seen under conditions with enhanced agrin cleavage were observed. The proportion of highly fragmented junctions was significantly increased in aged compared to adult animals (**Fig. 26f**). Only about 15% instead of about 70% of the presynaptic terminals were composed of 1 to 4 fragments, but 20 % of more than 12 (**Fig. 27a**). In average, presynapses of aged mice consisted of significantly more fragments than the adult junction (**Fig. 27b**). Similar results were obtained for the postsynaptic site (**Fig. 27c, d**). The fragmentation was less severe in aged than in Nto1 mice, resulting in a slight left shift of the distribution curves. This was in agreement with the muscle phenotype, which was more pronounced in Nto1 than in aged mice. The severity of NMJ fragmentation and muscle wasting nicely correlated. Unlike in transgenic animals, the synaptic area was not significantly changed in aged compared to adult mice (**Fig. 28**).

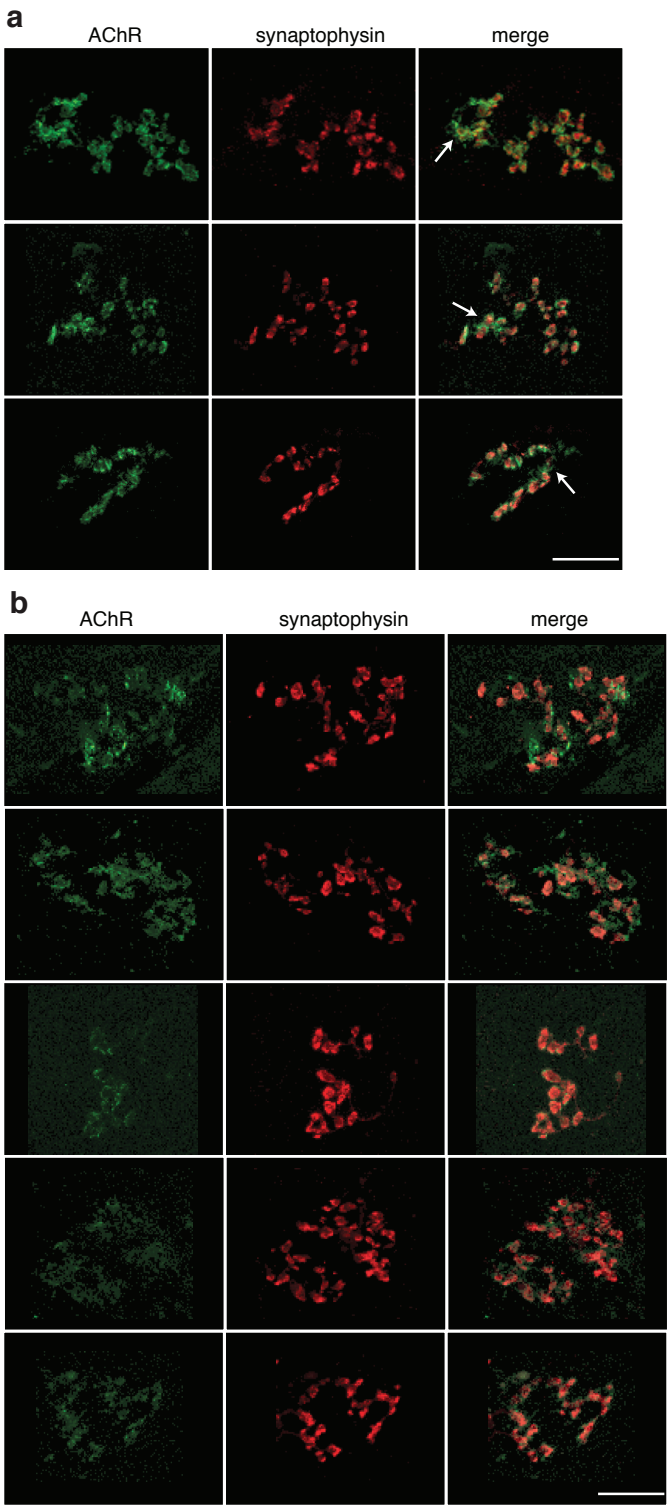
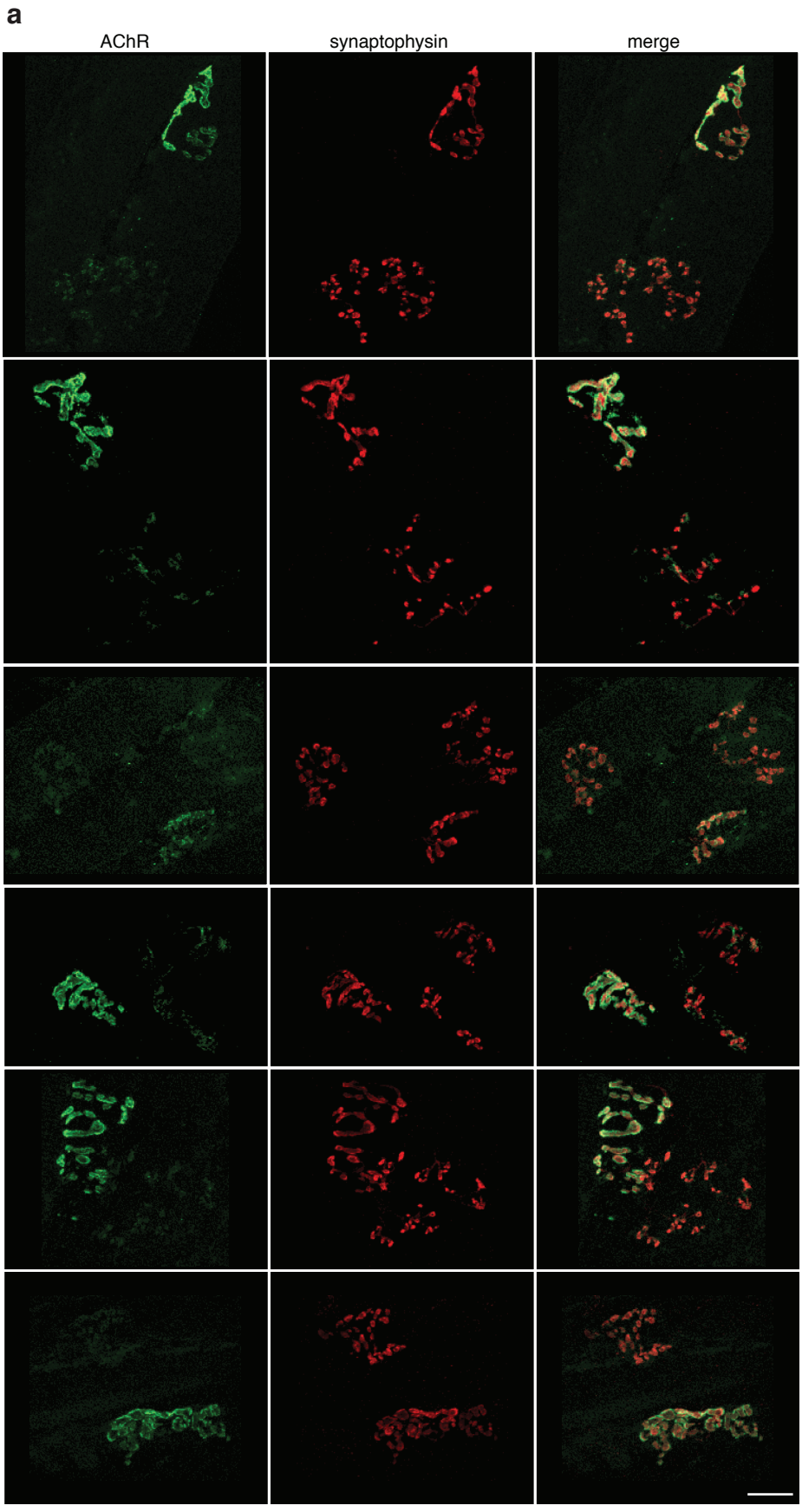


Figure 29 Partial misalignment of pre- and postsynptic sites may reflect an intial step on the way to NMJ dispersal. The NMJs on soleus muscles were visualized by synaptophysin antibodies (red) and Alexa-488 conjugated α-bungarotoxin (green). (**a**) Misalignment of a small portion of the pre- and postsynaptic sites was observed in a subset of NMJs on neurotrypsin transgenic muscles. Note the diffuse appearance of the postsynaptic signals in these regions (arrow). (**b**) Misalignment of a bigger portion of the pre- and postsynapse and weakening of the postsynaptic signals. Note the cloudy, diffuse appearance and the low intensity of the postsynaptic α-bungarotoxin signals in contrast to the well-defined presynaptic signals.

3.7.2 Fragmentation may lead to the disappearance of the NMJs

As mentioned above, colocalization of pre- and postsynaptic elements was still observed in the majority of the NMJs on the soleus muscles of transgenic mice (**Fig. 26b**). However, in about 5 % of the structures, this colocalization was not maintained. At some NMJs, only a small part of the presynapse was not directly opposed by postsynaptic receptors (**Fig. 29a**). At these sites, the postsynaptic signal had a diffuse, cloudy appearance. At more severely affected NMJs, the complete AChRs staining was diffuse and weak, whereas the presynaptic signal still was strong and precisely defined (**Fig. 29b**). Sometimes, the postsynaptic signal was hardly detectable (**Fig. 30a**). This finding suggested an ongoing dispersal of the postsynaptic receptor aggregates. In agreement with this hypothesis, endplates in which the postsynapse was completely absent were occasionally found (**Fig. 30b**). In these structures, highly fragmented presynaptic nerve endings were not opposed by α-bungarotoxin signals, indicating the absence of the postsynaptic AChR aggregates. Partial or total loss of AChR clusters was only found in the most fragmented NMJs (>12 fragments). These structures could represent final stages and indicate imminent disappearance of the NMJ. Excessive agrin cleavage appears to induce fragmentation, loss of synaptic area and eventually dispersal of NMJs. Thus, agrin might have a crucial role in the maintenance of NMJs during adulthood.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AChR
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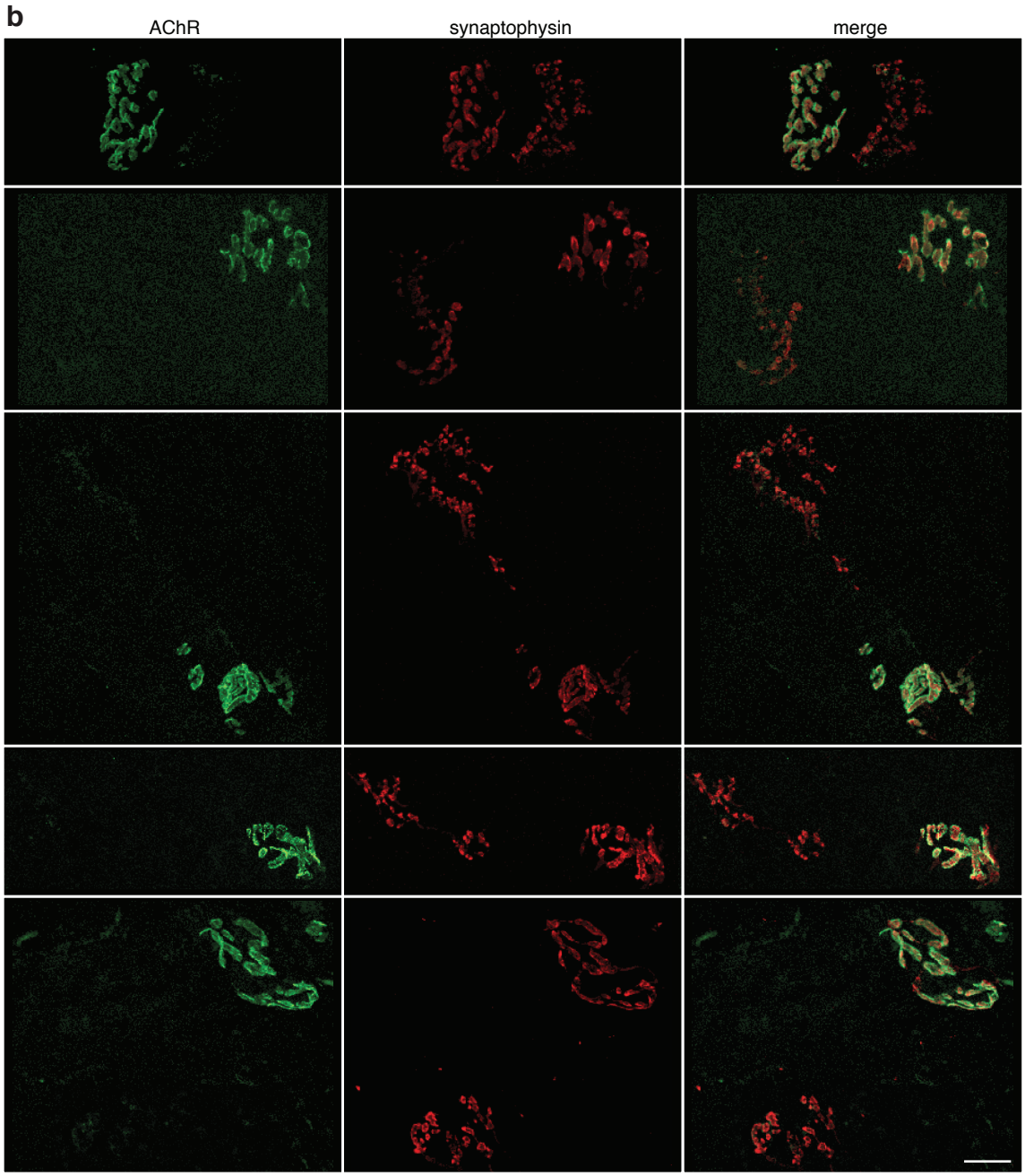


Figure 30 Agrin-cleavage induced fragmentation of the NMJ eventually lead to the disappearance of postsynaptic AChR aggregates. The NMJs on soleus muscles were visualized by synaptophysin antibodies (red) and Alexa-488 conjugated α-bungarotoxin (green). (**a**) Continuous dispersal of AChR aggregates resulted in almost complete absence of postsynaptic signals in a subset of NMJs on neurotrypsin transgenic muscles. Only weakly stained remnants of AChR clusters were left. As internal control, neighboring normally stained NMJs are included. (**b**) Remaining nerve terminals deprived of postsynaptic AChR aggregates were occasionally observed on neurotrypsin transgenic muscles, indicating the complete dispersal of the postsynaptic receptors. Note that partial or total loss of AChR aggregates only occurred in the most fragmented NMJs (>12 fragments). Scale bars, 20 μm.

Abandoned presynaptic terminals, which were not opposed by postsynaptic sites, were also occasionally found in aged animals (**Fig. 31**). Thus, age-dependent fragmentation may also result in the dispersal of the NMJ. Together, our results demonstrate a highly similar NMJ phenotype in mice exhibiting excessive agrin cleavage and in aged wild type mice. NMJ stability might be crucial for both neurotrypsin and aging induced sarcopenia.

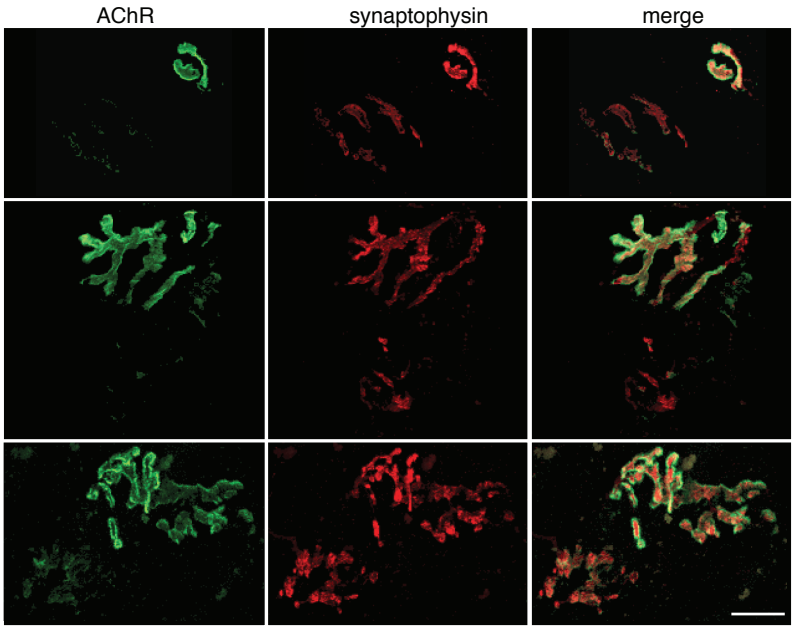


Figure 31 Rarely, presynaptic nerve endings (red) that are devoid of postsynaptic AChR aggregates (green) were also observed on the soleus muscles of aged animals. Scale bar, 20 µm.

Throughout the muscle of transgenic and aged mice, NMJs at different stages were found: unaffected, pretzel-like appearances, differently fragmented structures, and abandoned nerve terminals, which were not opposed by postsynaptic specializations. The availability of differentially affected NMJs may be represented by the muscle morphology. Atrophied and hypertrophied fibers that coexist within the same muscle could differ in the structure of their endplates; intact NMJs are capable to support muscle fibers to become hypertrophied, whereas severely fragmented NMJs may not be able to sustain synaptic transmission, resulting in atrophy of the associated muscle fibers.

3.7.3 Enhanced agrin cleavage at fragmented NMJs

Because the cleavage of agrin was thought to induce the dispersal of postsynaptic sites, we expected the disappearance of full-length agrin from highly fragmented, abandoned nerve terminals. To probe for the presence of agrin at fragmented NMJs triple stainings for synaptophysin, AChRs and agrin were prepared. Unfortunately, co-immunostainings for agrin and synaptophysin were not possible due to incompatibility of the antibodies. Instead, we used synaptophysin-GFP mice (spGFP) and crossed them with Nto1 mice. The fluorescently labeled transgenic synaptophysin revealed a similar staining pattern as the synaptophysin antibodies. However, the presynaptic sites appeared slightly punctuated, most likely due to accumulation of transgenic synaptophysin at certain places inside the presynaptic region. This accumulation appeared not to have any morphological or functional significance[638]. NMJs on the soleus muscle of spGFP and spGFP/Nto1 animals were stained for agrin-90 using the R132 antibody and for AChRs using fluorophore-conjugated α-bungarotoxin. In spGFP animals, agrin precisely colocalized with pre- and postsynaptic sites, representing its association with the basal lamina of the synaptic cleft (**Fig. 32a**). Surprisingly, fragmented NMJs in spGFP/Nto1 double transgenic animals still were positive for agrin, even when postsynaptic sites were absent (**Fig. 32b, c**). Thus, agrin remained aligned with abandoned presynaptic nerve endings. However, since an antibody against agrin-90 was used it could not be distinguished whether the signal corresponded to full length or to cleaved agrin. Agrin-90 may remain associated to the synaptic basal lamina after cleavage, because binding sites for α-dystroglycan, heparin, and integrins are present on this fragment[99]. To more directly analyze neurotrypsin-dependent agrin cleavage at the NMJ, the C-terminal cleavage fragment, agrin-22, was visualized using the G92 antibody together with agrin-90, AChR aggregates and synaptophysin. In spGFP animals, agrin-22 precisely colocalized with agrin-90 and the pre- and postsynaptic markers, suggesting the presence of full-length agrin at the basal lamina of these NMJs (**Fig. 33a**). In spGFP/Nto1 mice,

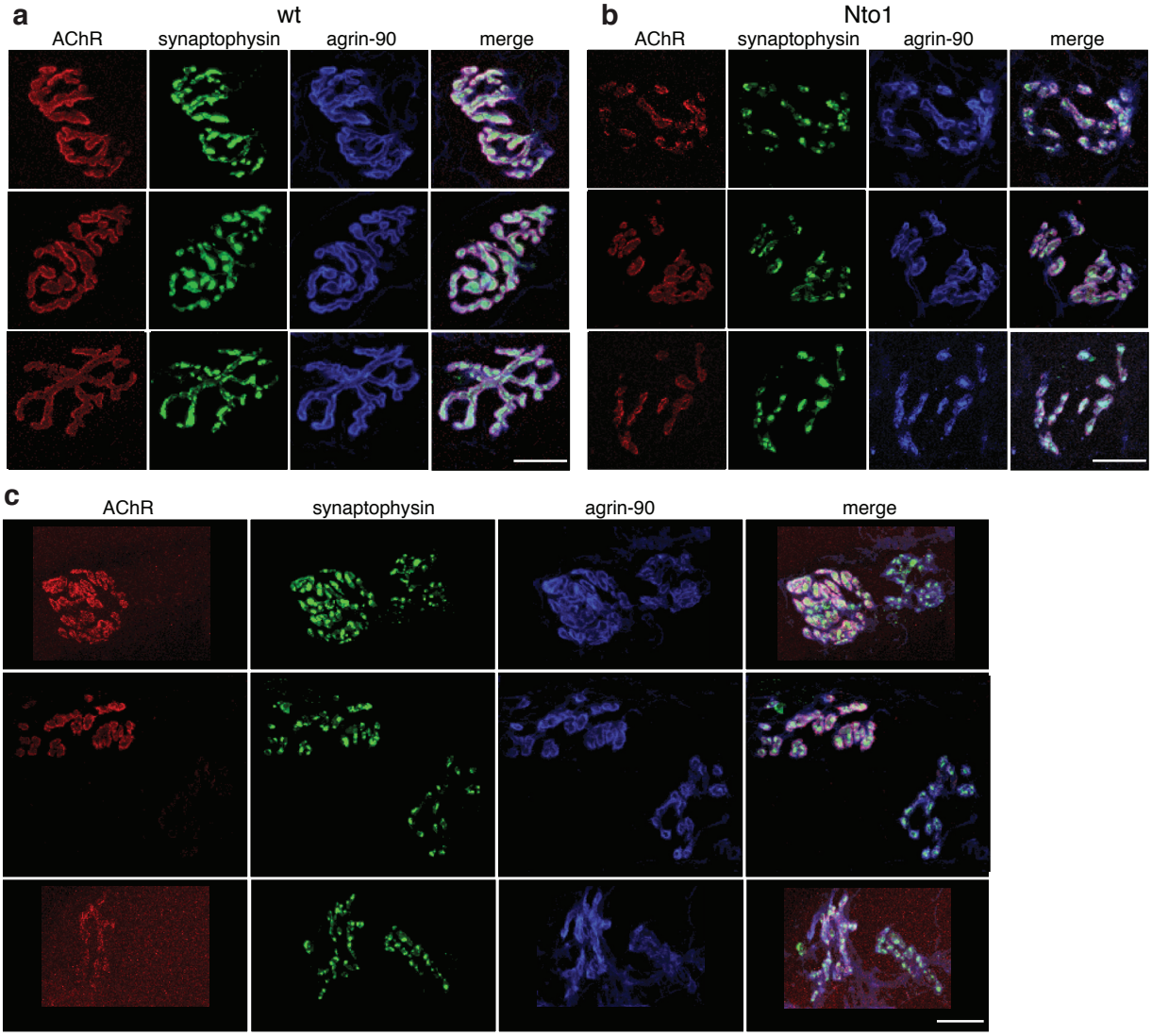
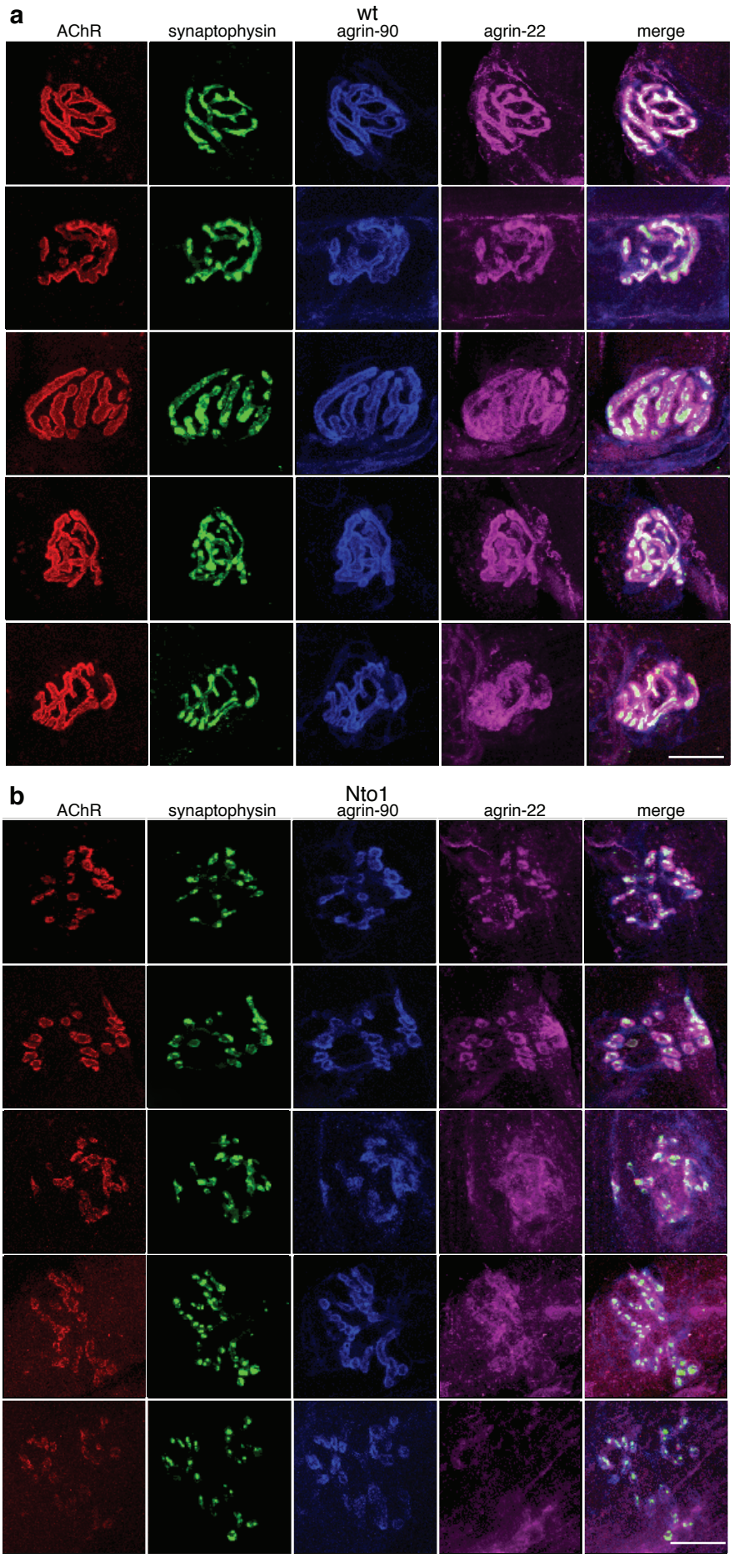


Figure 32 Agrin-90 remains attached to highly fragmented NMJs. Postsynaptic AChR aggregates were stained with tetramethylrhodamin (TMR)-conjugated α-bungarotoxin (red) and agrin-90 with R132 (blue) on soleus muscles of synaptophysin-GFP (green) transgenic mice. Note that the presynaptic synaptophysin-GFP label had a slightly punctuated pattern in these transgenic mice. (**a**) Agrin-90 precisely colocalized with both pre- and postsynaptic markers in wild type mice. (**b**) Fragmentation of the NMJs in Nto1 mice did not affect the alignment of agrin-90 with pre- and postsynaptic sites. (**c**) Even when postsynaptic AChRs were absent in heavily fragmented structures, the colocalization of agrin-90 and synaptophysin was preserved. In some of these cases, the signal strength of agrin-90 appeared to be reduced (bottom row). Scale bars, 20 µm

the signal for agrin-22 was clearly weaker in most of the endplates (**Fig. 33b**). In some cases, often characterized by severe fragmentation of pre- and postsynaptic sites, immunostaining for agrin-22 resulted in a diffuse signal at the place of the NMJ or even in the complete absence of any signal. Agrin-90 however was still present at those sites, indicating an increased cleavage of agrin and subsequent diffusion of agrin-22. Interestingly, weak or absent agrin-22 signals often co-occurred with weak α-bungarotoxin signals, which reflected ongoing dispersal of AChR aggregates. Thus, enhanced agrin cleavage, resulting in the disappearance of agrin-22 and hence, the loss of agrin's NMJ protecting function, could be responsible for the disappearance of the postsynaptic AChR clusters. In agreement with this hypothesis, agrin-22 has not been found in structures were the postsynaptic sites were completely absent (**Fig. 33c**). However, stainings for agrin-22 often showed high background, especially in cases with low signal intensities. Therefore,


a
wt
AChR
synaptophysin
agrin-90
agrin-22
merge
b
Nto1
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synaptophysin
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merge

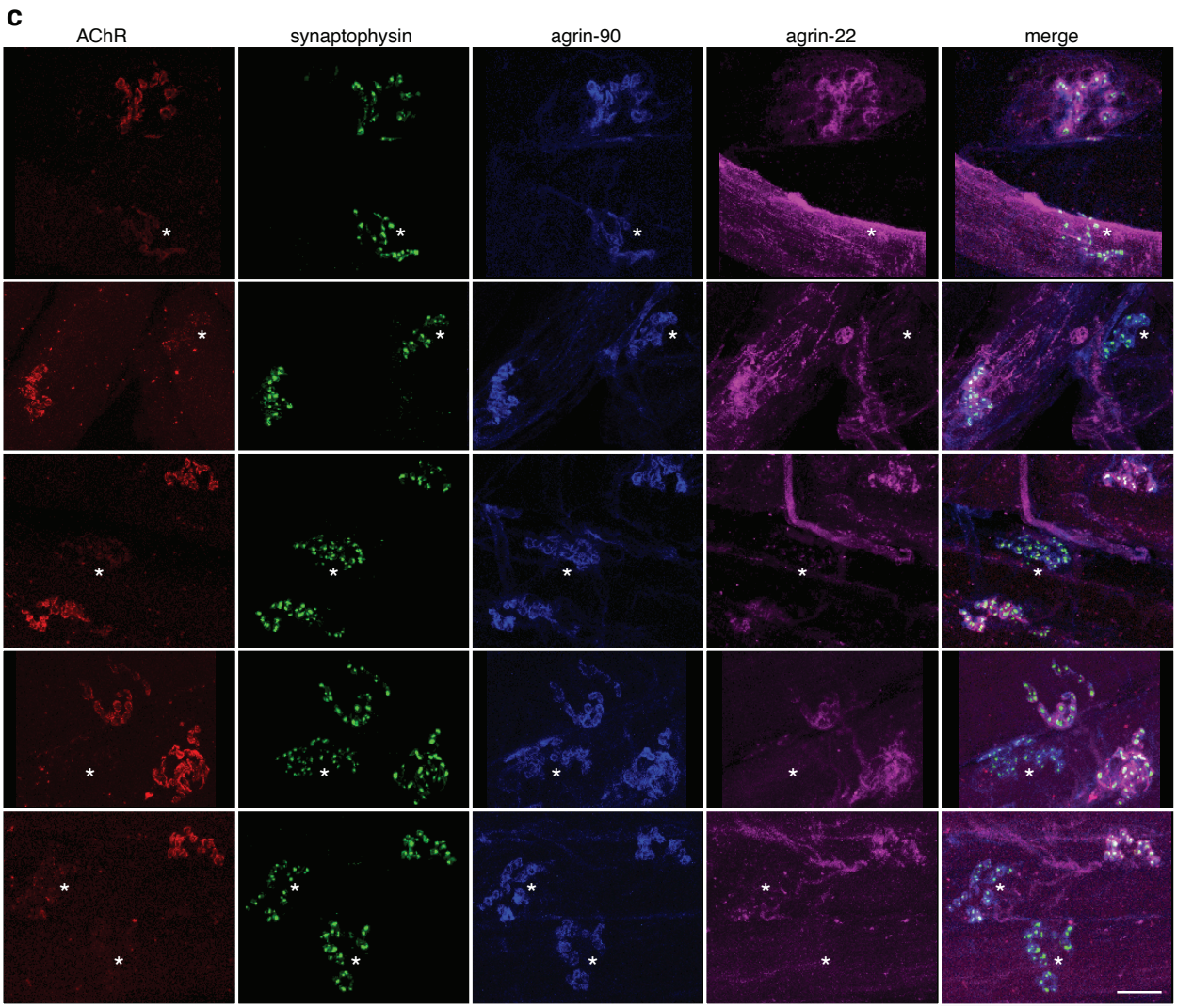
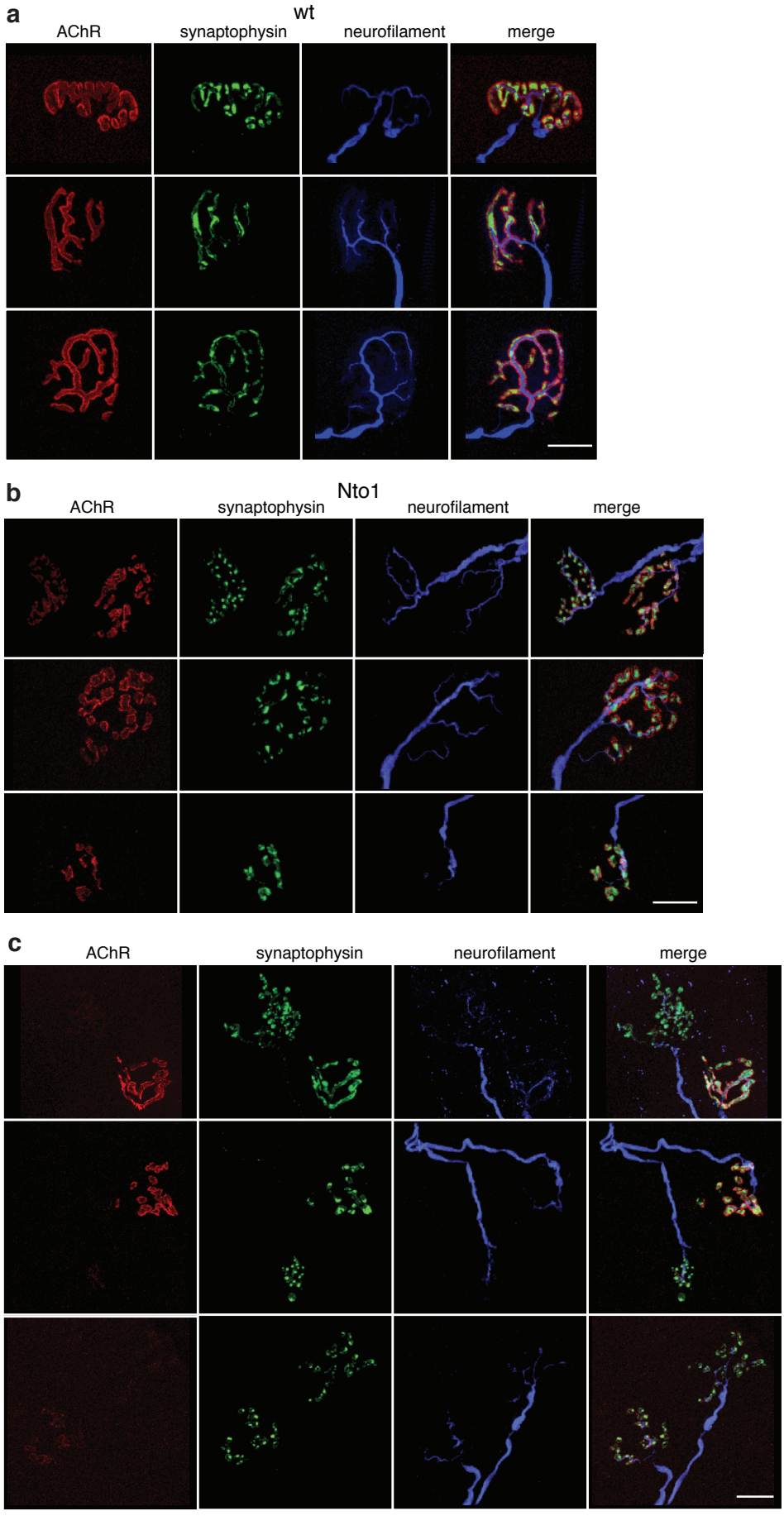


Figure 33 Visualization of agrin cleavage at highly fragmented NMJs. Triple stainings of agrin-90 with R132 (blue), agrin-22 with G92 (magenta), and AChR aggregates with TMR- conjugated α-bungarotoxin (red) on soleus muscles of synaptophysin-GFP (green) transgenic mice. (**a**) Agrin-90 as well as agrin-22 precisely colocalized with pre- and postsynaptic markers in wild type muscles, suggesting the presence of full-length agrin. (**b**) Following neurotrypsin overexpression in Nto1 mice, signals for agrin-22 appeared weak and diffuse in some NMJs, but still colocalized with agrin-90 in others. Enhanced cleavage of agrin followed by diffusion of agrin-22 at some NMJs might be the reason. Note that the absence of agrin-22 did not necessarily result in the immediate disappearance of the postsynaptic sites (bottom row). (**c**) Dispersal of postsynaptic AChR aggregates from heavily fragmented structures always co-occurred with the absence of agrin-22 (asterisks). Agrin-90, in contrast, still was associated with such structures. This observation suggested that the cleavage of agrin and the diffusion of agrin-22 could induce the dispersal of postsynaptic sites. Scale bars, 20 μm.

a better agrin-22 detection method would be needed to quantify these findings and to confirm the speculations.

3.7.4 Innervation of fragmented NMJs stays intact

To ensure that events at the NMJ and not axonal or motoneuronal degenerations were the cause of NMJ fragmentation, the innervation of transgenic endplates was studied. Fluorescently labeled antibodies against neurofilament, an intermediate filament specifically found in neurons, were used to stain nerve fibers. These experiments were also performed in spGFP transgenic mice to take advantage of the intrinsic presynaptic marker. Postsynaptic AChRs were stained


a
wt
AChR
synaptophysin
neurofilament
merge
b
Nto1
AChR
synaptophysin
neurofilament
merge
c
AChR
synaptophysin
neurofilament
merge

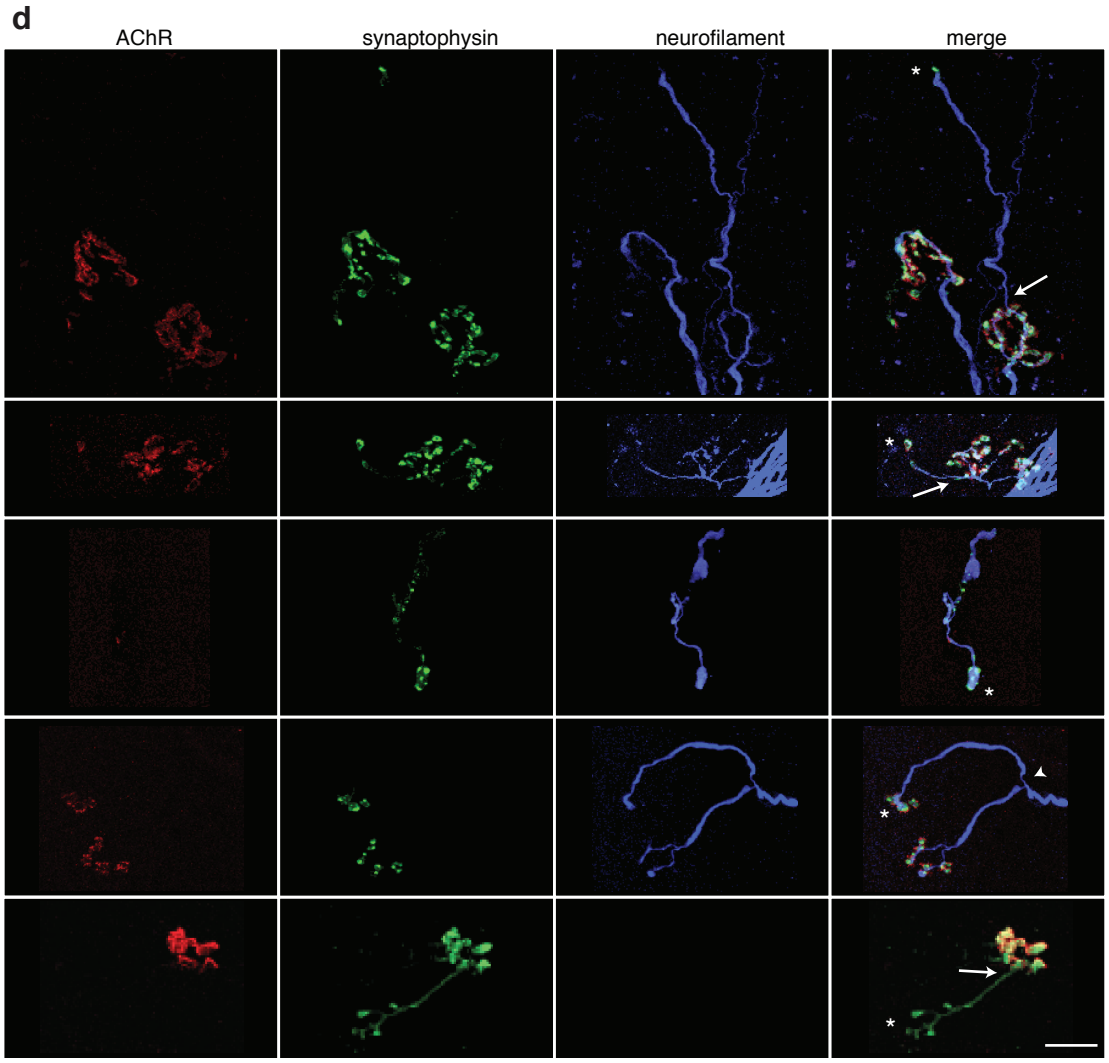


Figure 34 Innervation of highly fragmented NMJs stays intact. (**a-d**) NMJs on soleus muscles of synaptophysin-GFP (green) transgenic mice were stained for AChR aggregates with TMR-conjugated α-bungarotoxin (red) and for nerves with antibodies against neurofilament (blue). (**a**) In wild type muscles, single motor axons innervated the NMJs. (**b**) Heavily fragmented NMJs in Nto1 muscles were normally innervated. (**c**) The innervation stayed also intact when the postsynaptic AChR aggregates were dispersed. (**d**) Sprouting of motor axons in Nto1 animals from nerve terminals (arrows) or from the node of Ranvier (arrowhead). The outgrowing axons formed small immature synapses (asterisks), which were either positive for only synaptophysin, or for both synaptophysin and α-bungarotoxin. (**e**) Accumulation of small immature synapses in soleus muscles from Nto1 animals. Stainings with synaptophysin antibodies (red) and Alexa-488 conjugated α-bungarotoxin (green). Scale bars, 20 µm (**a-d**), 10 µm (**e**).

using fluorophore-conjugated α-bungarotoxin. The innervation pattern of fragmented NMJs in neurotrypsin transgenic animals appeared to be normal (**Fig. 34a, b**). Single nerve fibers innervated greatly fragmented NMJs as they did in normal pretzel-like endplates. Even structures, in which the postsynaptic sites were absent, were correctly innervated (**Fig. 34c**). This finding indicates that fragmentation and dispersal of the NMJs occurs despite correct innervation and thus, excludes axonal or motoneuronal degeneration as cause of NMJ deterioration. However, rarely, sprouts extending from an endplate (terminal sprouts) or an axon (nodal sprouts) to form new, small synaptic sites were observed, suggesting the reinervation of denervated fibers by neighboring motor axons (**Fig. 34d**). Furthermore, very small fluorescently labeled spots could represent the attempt of terminal axons, which have lost their contacts to the muscle, to form new synaptic sites (**Fig. 34e**). In summary, denervation and reinnervation events in conditions with increased agrin cleavage could be reflected by two processes: Motor axons that have lost their synaptic sites may regrow and search for new targets to innervate and denervated muscle fibers may induce sprouting of adjacent nerves to enforce reinnervation.

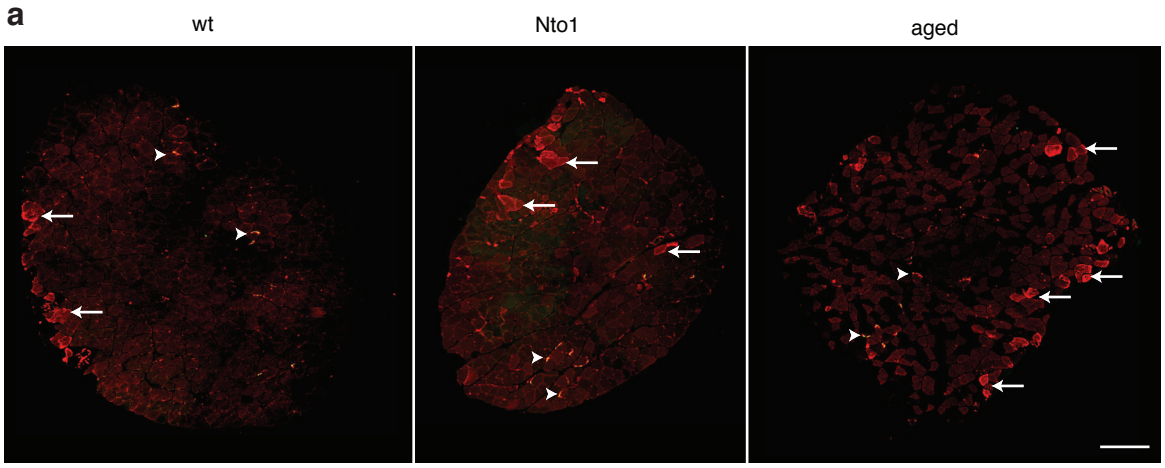
3.8 Analysis of denervated muscle fibers in the soleus muscle

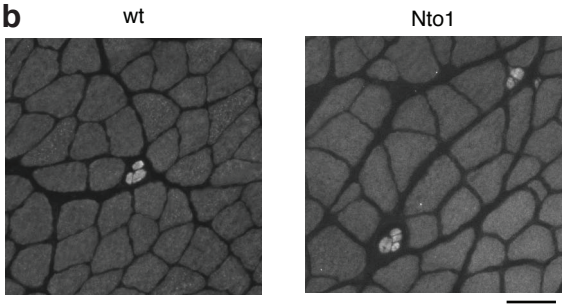
Appearance of angular muscle fibers, fragmentation of the NMJs, and axonal sprouting all indicated that denervation of muscle fibers might cause their atrophy and degeneration. We tried to directly detect denervated muscle fibers in the soleus muscle to confirm this hypothesis. Several characteristics of denervated muscle fibers can be used to detect them on muscle cross-sections, in single muscle fiber preparations or by Western blotting on muscle protein extracts.

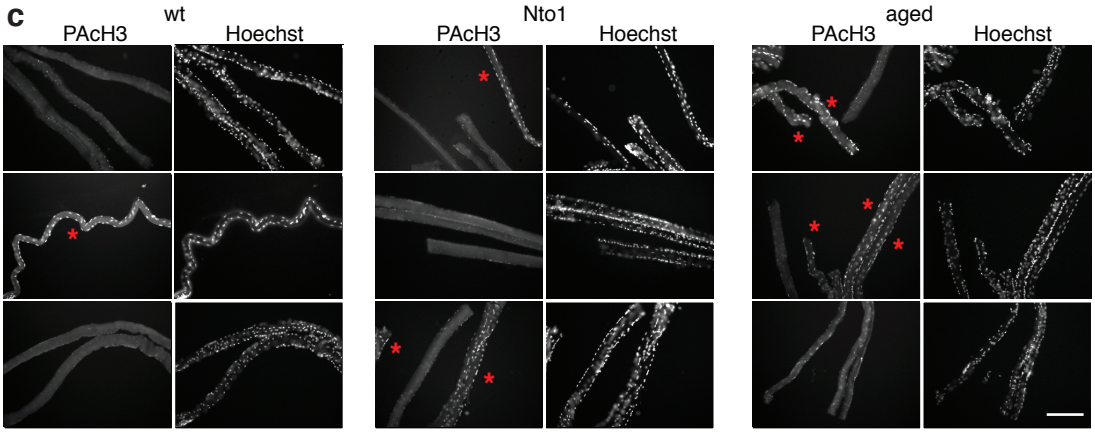
It has been reported that the neural cell adhesion molecule (NCAM) is redistributed upon denervation of muscle fibers[208,257]. Normally, NCAM resided at the NMJ, but after denervation, it appeared all over the fiber membrane. We co-stained muscle cross-sections with an antibody against NCAM and α-bungarotoxin (**Fig. 35a**). Fibers, which were single labeled for NCAM, were counted as denervated. The NMJs served as internal control, because they contain NCAMs and AChRs and, hence, should be stained with both NCAM antibodies and α-bungarotoxin. The endplates on all muscle sections were clearly double stained, confirming the specificity of the NCAM antibody. Neurotrypsin overexpression and aging were associated with a slightly higher proportion of denervated muscle fibers but the differences were not significant (**Fig. 35d**). However, we faced several problems with this method that put its reliability into question. First, the variation between cross-sections from the same muscle was considerable. Second, the labeled fibers were often clustered at the boarder of the muscle, and third, mechanically damaged fibers regularly were NCAM positive. Unfortunately, an explicit negative control (all fibers innervated), as well as a positive control (all fibers denervated) was missing. Thus, an influence of the preparation on the immunostainings could not be completely excluded. The treatment of the cross-sections during staining or especially cutting was suspected to lead to false positive results.

A second method was based on the findings, that the tetrodotoxin-resistant sodium channel ($Na_v1.5$) is expressed after denervation, but absent in innervated adult muscle[330]. An antibody against this sodium channel was applied on cross-sections from transgenic animals (**Fig. 35b**). However, no immunostained fibers were detected in transgenic or wild type muscles; only some fragmented, degenerated remains of fibers were labeled. Whether the antibody was not working on immunostainings or whether all fibers were innervated could not be answered, due to the absence of adequate controls.

Another possibility to detect denervated muscle fibers came from observation of the histone acetylation pattern of myonuclei. The acetylation and phosphorylation of histone H3 was increased after denervation[643]. Single muscle fibers were prepared from the soleus muscle to analyze the

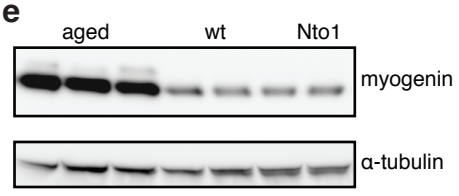






d

	NCAM positive fibers (%)		PAcH3 postive fibers (%)	
	n		n	
wt	5	1.69 ± 0.40	8	27.01 ± 8.22
Nto1	6	2.44 ± 0.90	4	32.56 ± 5.72
aged	4	2.04 ± 0.43	4	31.98 ± 6.52



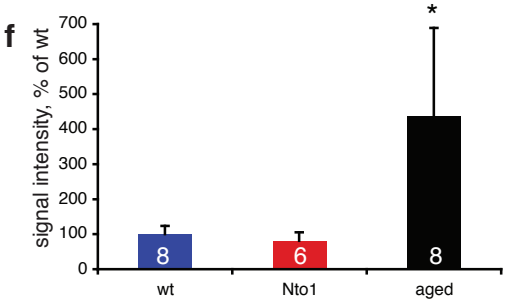


Figure 35 Analysis of denervated muscle fibers in transgenic and aged soleus muscle did not lead to reliable results. (**a**) Overlays of soleus muscle cross-sections from adult wild type, Nto1, and aged animals stained for NCAM (red) and AChR (green). Arrows indicate NCAM positive fibers and arrowheads indicate double stained NMJs. (**b**) Soleus muscle cross-sections immunostained for tetrodotoxin-resistant sodium channels ($Na_v1.5$). Only fragmented remnants of fibers were labeled. (**c**) Single soleus muscle fiber stained for phosphorylated and acetylated histone H3 (PAcH3).

The nuclear Hoechst stain was used to mark the myonuclei. Muscle fibers with PAcH3-positive nuclei (asterisks) were observed in wild type, Nto1 and aged muscles. (**d**) Quantification of the percentage of NCAM and PAcH3 positive fibers in wild type, neurotrypsin-transgenic and aged muscles. Significant differences were not detected. All fibers of each muscle were measured for all quantifications. n indicates the number of animals. Data represent mean ± s.d. (**e**) Western blot of gastrocnemius muscle extracts probed for myogenin. Immunoreactivity for myogenin was increased in aged muscles. α-tubulin was used as loading control. (**f**) Quantification of myogenin signal intensities. In aged muscles the amount of myogenin was more than 4-fold increased compared to adult wild types (wild type value set to 100%). Neurotrypsin overexpression did not influence myogenin expression. The number of animals for each group is indicated inside each bar. Error bars represent s.d. *P < 0.05 compared to wild type.
Scale bars, 0.1 mm (**a**), 50 μm (**b**), 0.2 mm (**c**).

immunostaining of the nuclei. After fixation, very thin forceps were used to tease the muscle into single fibers. An antibody against acetylated and phosphorylated histone H3 was applied (αPAcH3). The nucleic acid stain Hoechst was used as nuclear marker and staining intensity control. Fibers with PAcH3 positive nuclei were frequently observed (**Fig. 35c**). However, in some cases it was quite difficult to judge whether the nuclei were stained or not, since the intensities did not differ greatly. Quantitative analysis revealed a non-significant increase in PAcH3 positive fibers in neurotrypsin transgenic and in aged animals (**Fig. 35d**). However, the control level of positive fibers in adult wild type muscles was very high (about 27 %). Previous studies have only found 1-2% of denervated fibers in adult animals[208,257,330], indicating that a lot of the PAcH3 positive fibers may not represent denervated fibers. Moreover, the variation between different animals, regardless whether transgenic or not, was considerable. Together, this method may not be feasible to detect some denervated fibers in whole muscle preparations. It might be possible to find differences between normally innervated and fully denervated muscles but not relatively small changes in a slightly denervated muscle.
The expression of the transcription factor myogenin, an activator of muscle specific genes, has been shown to be upregulated in denervated[607,608] and regenerating skeletal muscles[604-606]. Because the α-myogenin antibody was not working for immunostainings, Western blotting on gastrocnemius muscle extracts was performed (**Fig. 35e**). A signal for myogenin at 25 kDa was detected. Signal intensities in neurotrypsin transgenic and wild type muscle extracts were not systematically different, although considerable variations between individual animals were observed. The upregulation of myogenin in few denervated muscle fibers may not be enough to significantly alter the myogenin level in the whole muscle. Moreover, it has been shown that myogenin up- and downregulation is a fast process and can be accomplished in days[610]. In aged animals, however, the signal for myogenin was more than 4-fold increased (**Fig. 35f**). Whether this is due to denervated fibers, ongoing regeneration of the muscle or other age-related changes in protein synthesis is currently unclear.
Different methods for the detection of denervated muscle fibers were used, but none of them led to significant and reliable results. Technical problems with the methods, like for

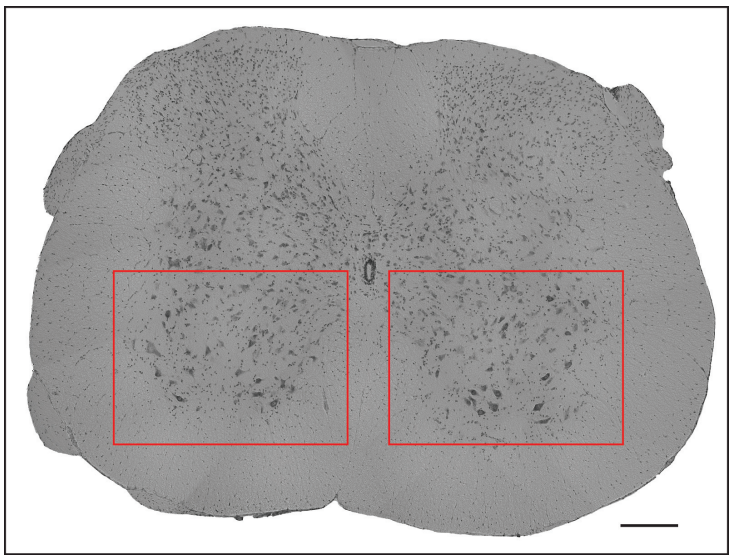
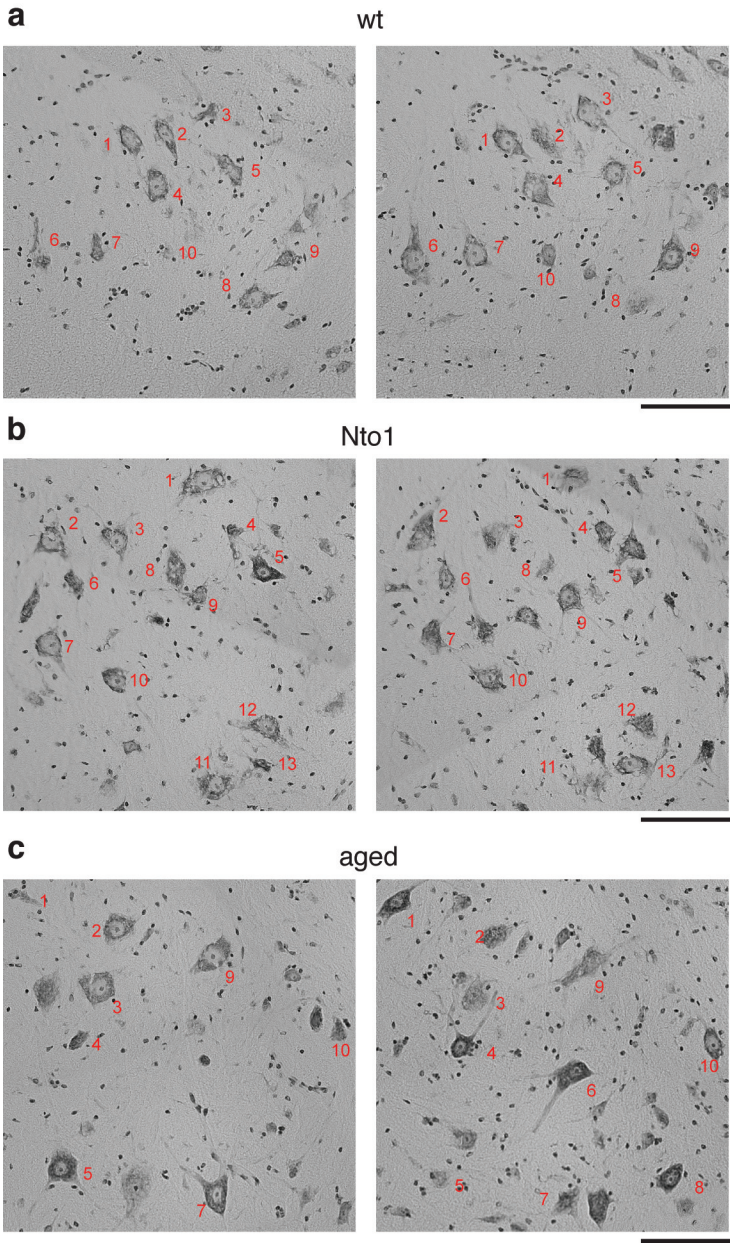


Figure 36 Cresyl violet stained spinal cord cross-section. The motoneurons can be found in the ventral horns (red boxes). Scale bar, 0.2 mm.

example the absence of good controls, were one of the reasons. Furthermore, the actual amount of denervated muscle fibers could be very low in transgenic muscles, since the time a fiber remains denervated, before it gets reinnervated or degenerated, might be relatively short. A low number of denervated fibers would be difficult to detect with the applied methods.

3.9 The number of motoneurons is not altered in neurotrypsin-overexpressing or aged mice

The fragmentation of the NMJ results in disconnection between motoneuron and muscle fiber. Muscle fibers are extensively lost, but the fate of the motoneurons remains elusive. We analyzed the number of motoneurons in spinal cord cross-sections to address this question. The spinal



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	no of motoneurons		
	wt	Nto1	% of wt
pair 1	270	242	90
pair 2	286	283	99
pair 3	236	242	103
pair 4	270	267	99
pair 5	244	248	102
pair 6	208	219	105
			99

e

	no of motoneurons		
	adult	aged	% of adult
pair 1	283	286	101
pair 2	214	198	93
pair 3	212	212	100
pair 4	212	209	99
pair 5	246	237	96
			98

Figure 37 The number of motoneurons in the spinal cord is not affected by neurotrypsin overexpression or aging. (**a-c**) Cresyl violet stainings of two consecutive spinal cord cross sections from a wild type (**a**), a neurotrypsin transgenic (**b**) and an aged mouse (**c**). Motoneurons were identified by their size, their position, and the existence of a clear nucleus. They were carefully traced through the sections. Numbers identify the same motoneurons in serial sections. (**e**) Comparison of the number of motoneurons in six pairs of mice, each including a wild type and neurotrypsin-overexpressing animal from line Nto1, which were processed in parallel. The motoneurons were counted in ten consecutive sections at four different positions in the lumbar and sacral part of the spinal cord. No significance difference was detected. (**f**) Comparison of the number of motoneurons in 5 pairs of mice, each including an adult and an aged animal. No significant difference was detected. Scale bars, 0.1 mm.

cord of a neurotrypsin-overexpressing and a wild type mouse were aligned and processed in parallel. Ten serial 12 µm cross-sections were cut at four different positions in the lumbar and sacral regions of the spinal cords and stained with cresyl violet (**Fig. 36**). The motoneurons were identified by their size, their position in the ventral horns and the presence of a clearly identifiable nucleus. They were counted throughout the 10 serial sections at 4 different positions in the lumbar and sacral part of the spinal cord (**Fig. 37a-c**). Thereby, the individual motoneurons were traced through the sections, to make sure that every motoneuron was only counted once. The total number of motoneurons in these four stacks of 10 sections was compared between the two spinal cords, which were processed in parallel, and relative differences were calculated. Such a pairwise comparison didn't reveal any difference in the number of motoneurons between neurotrypsin transgenic and wild type mice (**Fig. 37d**). Comparison of aged and adult animals did not reveal any differences either (**Fig. 37e**). Thus, neither in neurotrypsin-overexpressing nor in aged wild type animals was NMJ fragmentation and muscle fiber loss preceded or accompanied by a loss of motoneurons.

3.10 The role of neurotrypsin and agrin cleavage in age-associated muscle wasting

3.10.1 Neurotrypsin is not essential for the development of sacropenia

Our results suggest an important role of the NMJ stability in the etiology of sarcopenia. A very severe sarcopenia-like muscle wasting can be established by destabilizing the NMJ, without affecting axons or motoneurons. Therefore, NMJ destabilizing (neurotrypsin) or stabilizing (agrin) agents could be crucial players in the progressive age-associated decline of skeletal muscle mass and function. The balance between neurotrypsin and agrin could determine the stability of the

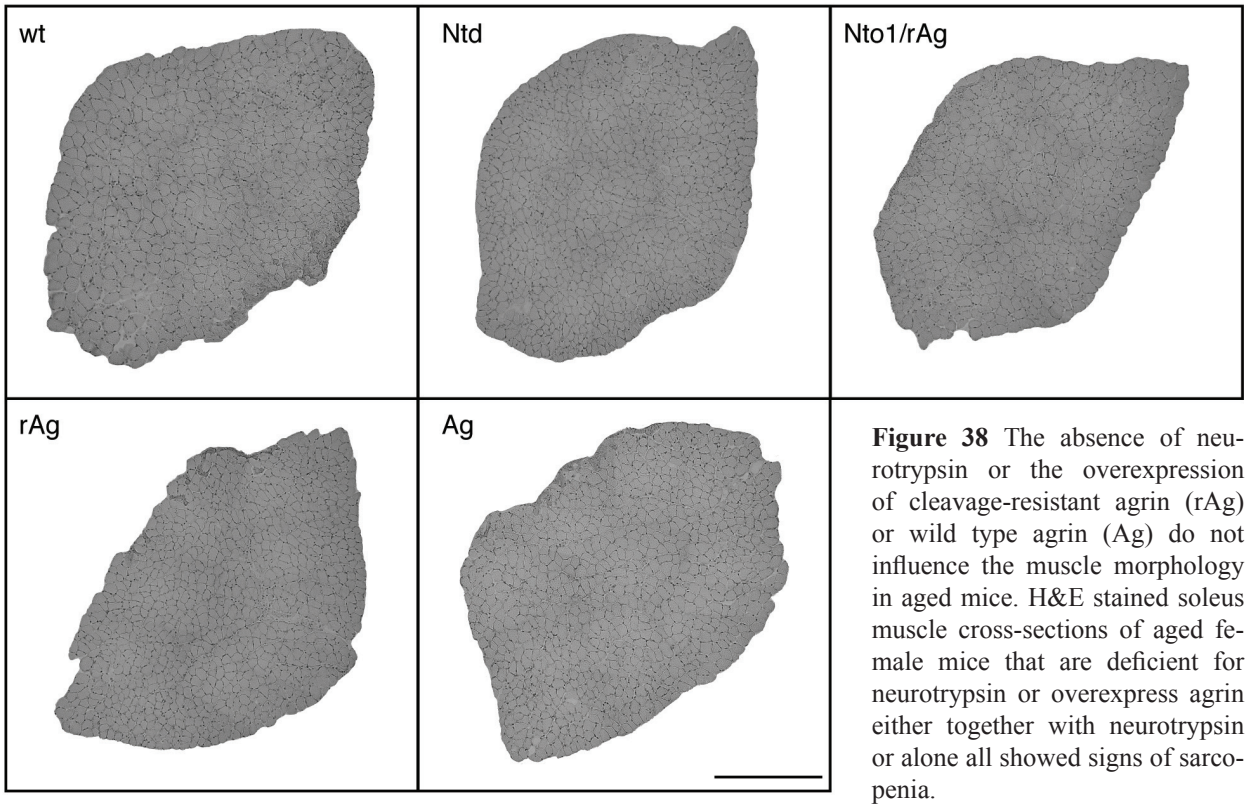

Figure 38 The absence of neurotrypsin or the overexpression of cleavage-resistant agrin (rAg) or wild type agrin (Ag) do not influence the muscle morphology in aged mice. H&E stained soleus muscle cross-sections of aged female mice that are deficient for neurotrypsin or overexpress agrin either together with neurotrypsin or alone all showed signs of sarcopenia.

NMJ, thereby influencing the ability of a motoneuron to establish a stable contact to its target muscle fiber.
To address the involvement of neurotrypsin-dependent agrin cleavage in the etiology of sarcopenia,

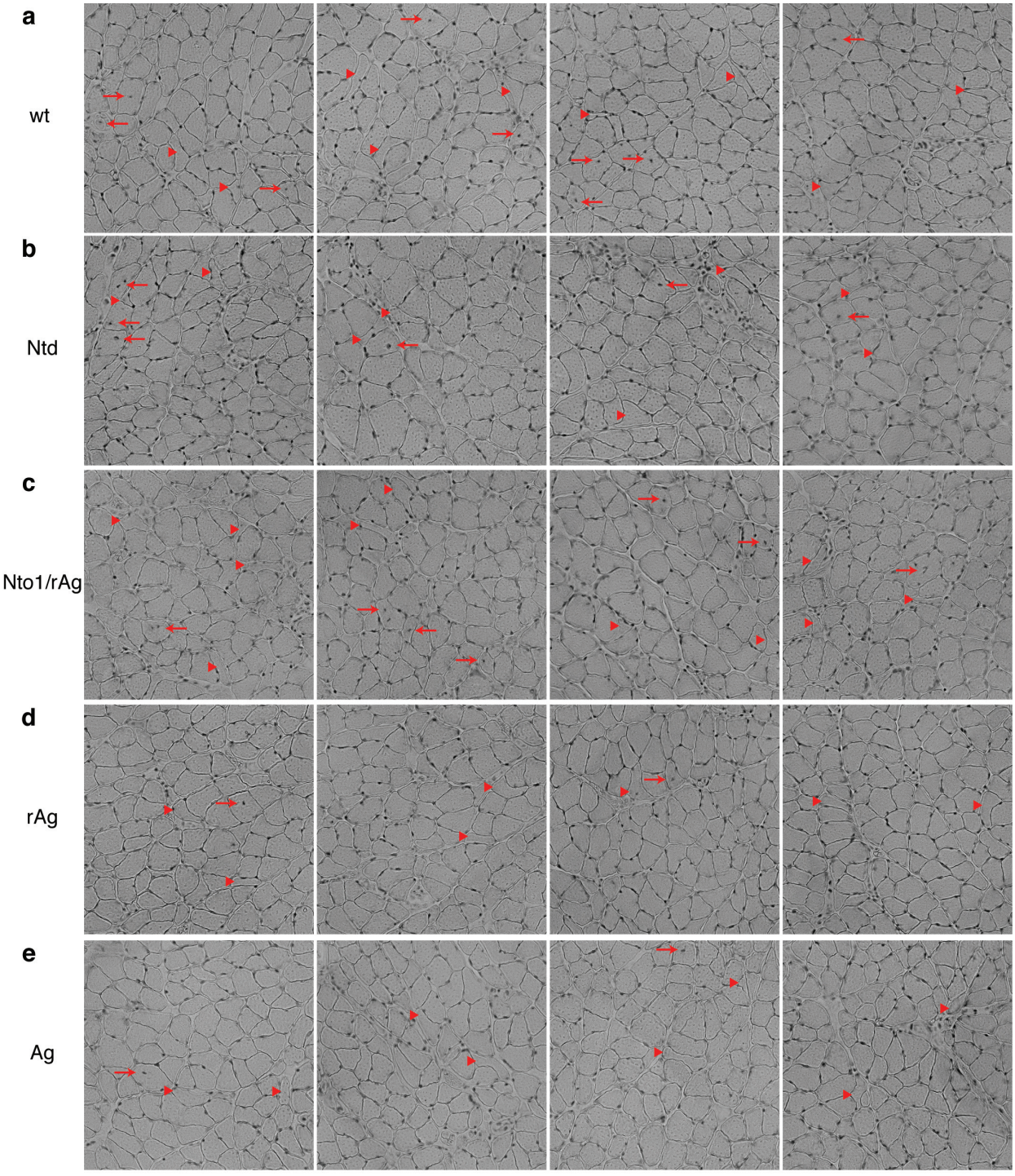

Figure 39 The absence of neurotrypsin and the overexpression of neurotrypsin-resistant agrin (rAg) or wild type agrin (Ag) do not prevent age-associated sarcopenia. (**a-e**) H&E stained cross-sections of aged female wt (**a**), Ntd (**b**), Nto1/rAg (**c**), rAg (**d**), and Ag (**e**) mice showed similar age-dependent alterations characteristic for sarcopenia. Hypertrophied and atrophied as well as centrally nucleated (arrows) and angular fibers (arrowheads) were observed. Note that muscles from both cleavage-resistant and wild type agrin-overexpressing animals appeared to exhibit less of the sarcopenic features. Scale bar, 0.1 mm.

Table VII
Morphology of aged muscles

		number of fibers		fiber CSA (μm^2)		centrally nucleated fibers (%)			fiber shape (circularity)		type I fibers (%)	
line	genotype	n		n		n			n		n	
Ntd	wt	7	803 ± 40	5	1570 ± 99	5	1.79 ± 0.49		5	0.795 ± 0.006	5	55 ± 3
	ko	9	826 ± 55	6	1572 ± 91	5	1.78 ± 0.21		6	0.795 ± 0.011	7	53 ± 5
Nto1	wt	8	805 ± 57	7	1557 ± 91	7	1.70 ± 0.49		7	0.797 ± 0.010	7	53 ± 4
x rAg	Nt/rAg	6	782 ± 121	6	1563 ± 135	6	1.76 ± 0.62		6	0.800 ± 0.010	5	49 ± 6
x rAg	rAg	4	857 ± 44	4	1466 ± 184	4	0.75 ± 0.30	*	4	0.805 ± 0.008	4	56 ± 2
x Ag	Ag	5	854 ± 17	5	1480 ± 66	5	0.81 ± 0.21	*	5	0.807 ± 0.005	4	55 ± 5

The absence of neurotrypsin or the overexpression of resistant agrin do not significantly improve muscle morphology in aged muscles. Comparison of the muscle morphology of aged female wt, Ntd, Nto1/rAg, rAg, and Ag mice. Note the slight differences of rAg- and Ag-overexpressing animals compared to wild types in regard to fiber number, fiber CSA, centrally nucleated fibers, fiber shape and percentage of type I fibers. The difference in the relative amount of fibers possessing centralized nuclei was statistically significant. n indicates the number of animals for each group. Data represent mean ± s.d. *P < 0.05 compared to wild type littermates.

aged (24 months-old) neurotrypsin deficient mice (Ntd) were analyzed. They were not distinguishable from aged wild type animals, neither from the appearance nor from the muscle morphology (**Fig. 38, 39a, b**). Aged Ntd mice similarly suffered from muscle fiber loss, and exhibited the same heterogeneity of muscle fiber thickness (**Table VII**, **Fig. 40**). Centrally located nuclei and angular muscle fibers were observed with a similar frequency than in aged wild type mice (**Table VII**). Finally, aged Ntd muscles exhibited type I fiber preference and fiber type grouping to the same extent as aged wild type muscles (**Fig. 41, 42a, b, Table VII**). These findings indicate that neurotrypsin does not play an important role in the etiology of sarcopenia. Moreover, the accumulation of agrin-90 in aged spinal cords appears not to be a necessary condition for sarcopenia, since it does not occur in Ntd animals. Nevertheless, neurotrypsin-independent agrin cleavage or degradation at the NMJ could be involved in the pathogenic process leading to sarcopenia. The absence of any agrin cleavage fragments in spinal cord extracts of Ntd mice does not exclude the existence of another agrin cleaving process at the NMJ, because agrin processing in the synaptic cleft can't be detected in spinal cord extracts. Direct analysis of agrin cleavage at the NMJ was

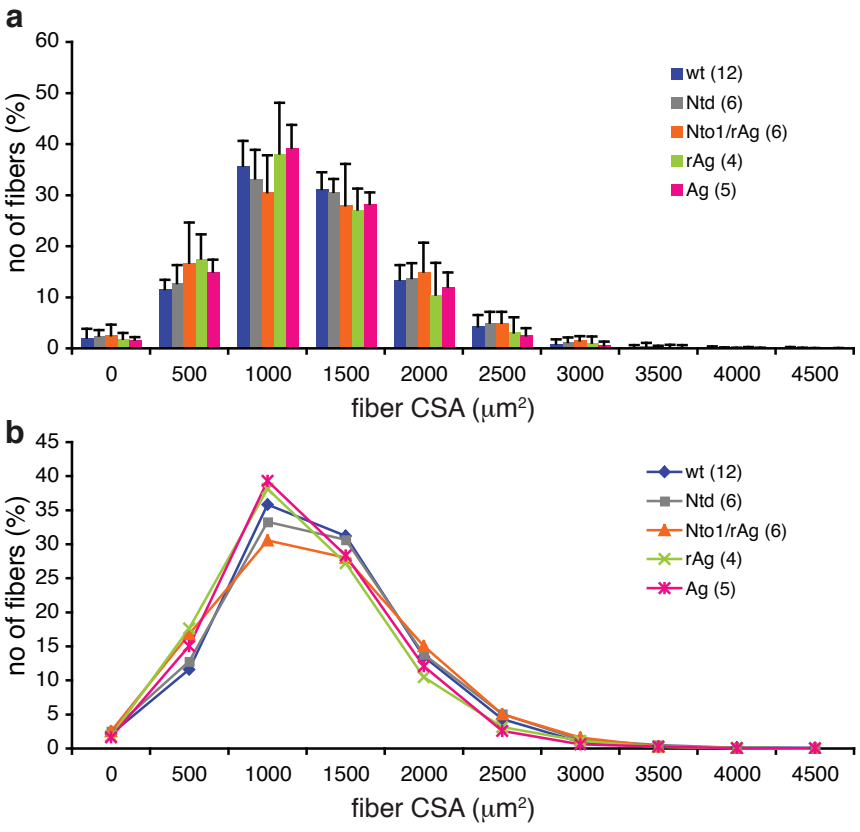

Figure 40 Frequency histogram of the fiber size in soleus muscle of aged female Ntd, Nto1/rAg, rAg, and Ag mice. No significant difference was detected but fiber thickness was slightly less heterogeneous in rAg- and Ag-overexpressing muscles. All fibers of each muscle were measured for all quantifications. The number of animals for each group is indicated in parentheses. Error bars represent s.d.

not possible since spinal cord extracts do not include the NMJs and in whole muscle extracts, the amount of NMJ constituents is by far too low. Alternatively, agrin could also be completely degraded, not leaving detectable fragments behind. So far, it was not possible to confirm or exclude age-associated enhancement of agrin cleavage at the NMJ.

3.10.2 Agrin cleavage at the neurotrypsin-dependent cleavage sites may be dispensable for sarcopenia

Since enhanced agrin cleavage precociously induced sarcopenia in adult mice, we speculated that agrin cleavage could be involved in the etiology of sarcopenia. Thereby, a second agrin cleaving protease, distinct from but related to neurotrypsin might be important. It has been shown that the concomitant overexpression of a neurotrypsin-resistant agrin variant and neurotrypsin delayed the NMJ maturation. In absence of neurotrypsin, in contrast, the NMJ maturation was normal (Bolliger et al., in preparation). To explain this finding, a protease with the same site specificity as neurotrypsin has been proposed. This protease would not be expressed in the central nervous system but would have access to the NMJ, where it can cleave agrin. Consequently, the overexpression of the proteolysis-resistant agrin variant (rAg) could prevent agrin cleavage by this protease in age, delaying or preventing sarcopenia. Alternative to cleavage, reduced agrin synthesis in senescence could also result in a lower amount of agrin's signaling component. Also this process could be counteracted by transgenic overexpression of agrin. Therefore, we analyzed aged Nto1/rAg-, rAg-, and Ag-overexpressing animals at the age of 24 months for the occurrence of sarcopenia. We found that these mice exhibited the histopathological features of sarcopenia to almost the same extent as wild type mice, including a reduced number of fibers, increased heterogeneity of fiber thickness, angular fibers, centralized nuclei, and increased proportion of type I fibers together with fiber type grouping (**Fig. 38, 39c-e, 40, 41, 42c-e**). Significant differences were only detected in the relative amount of centrally nucleated muscle fibers, which was decreased in aged rAg- and

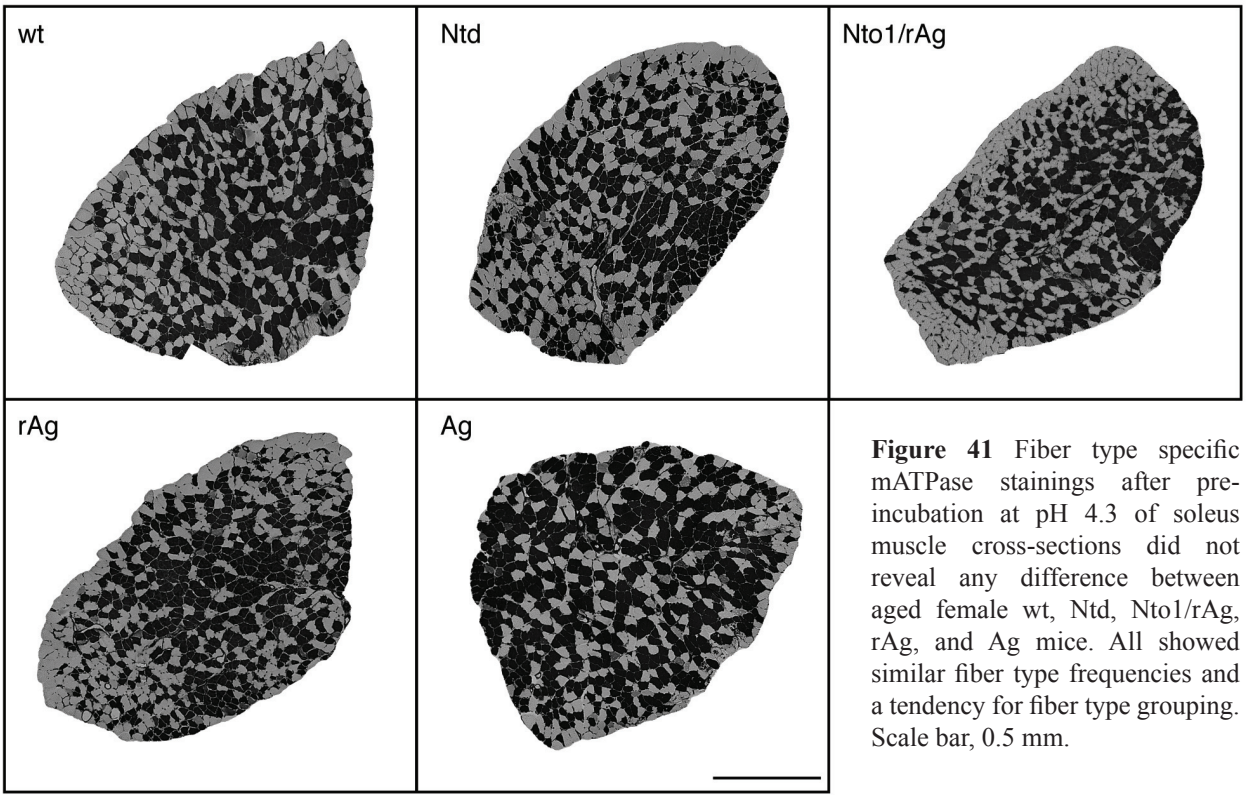

Figure 41 Fiber type specific mATPase stainings after pre-incubation at pH 4.3 of soleus muscle cross-sections did not reveal any difference between aged female wt, Ntd, Nto1/rAg, rAg, and Ag mice. All showed similar fiber type frequencies and a tendency for fiber type grouping. Scale bar, 0.5 mm.

Ag-overexpressing animals compared to wild type littermates (**Table VII**). However, changes in several other morphological parameters were observed, even though these changes were statistically not significant. The number of fibers was increased in senescent rAg- and Ag-overexpressing mice compared to wild type littermates. As a consequence, the compensatory hypertrophy was less explicit in these animals, resulting in a lower mean fiber CSA. Interestingly, the type I fiber

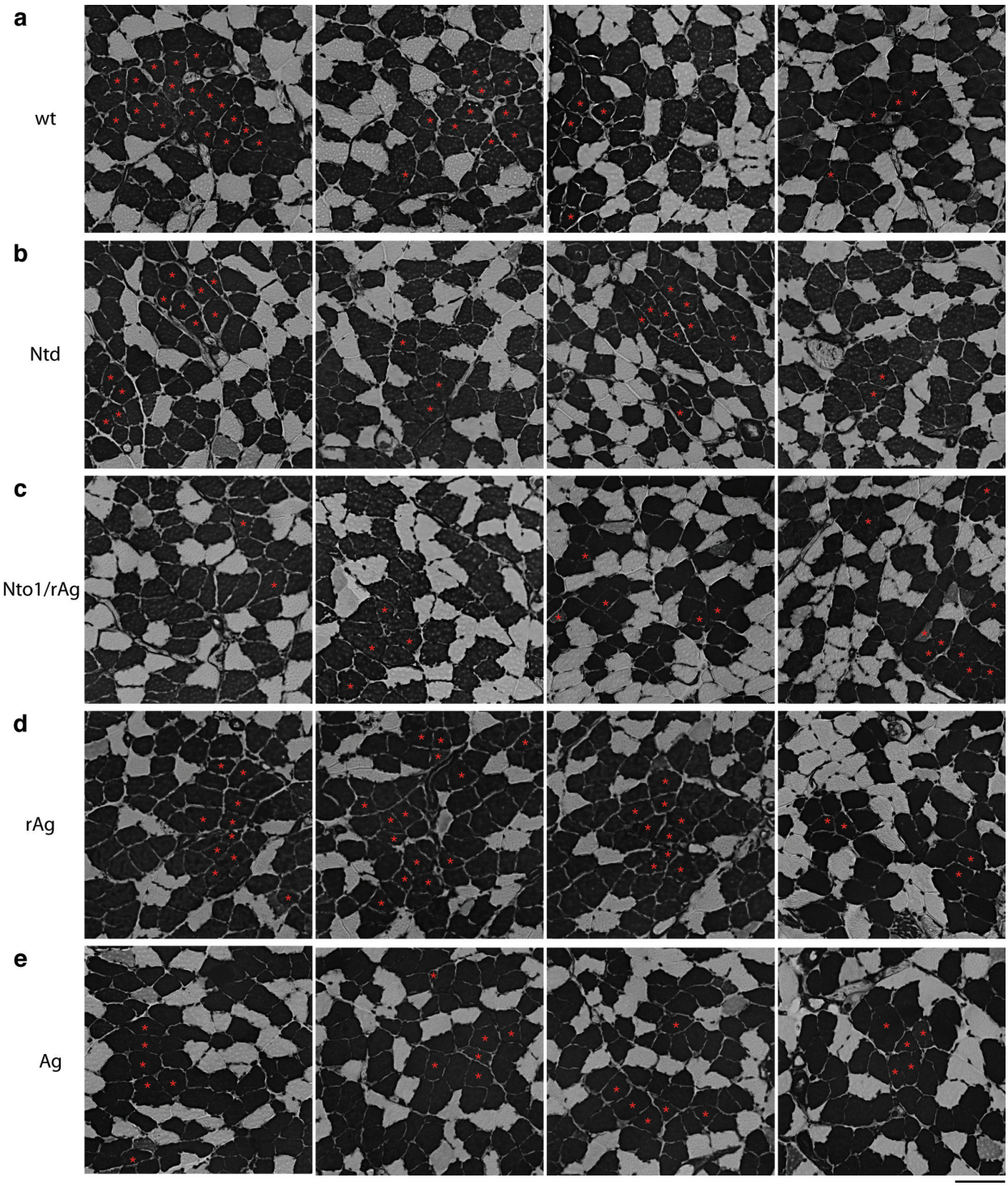

Figure 42 Cross-sections of female soleus muscles from aged wt (**a**), Ntd (**b**), Nto1/rAg (**c**), rAg (**d**), and Ag (**e**) mice stained for mATPase activity after preincubation at pH 4.3. The proportion of darkly stained type I fibers was increased in all aged animals compared to adults. Enclosed fibers (asterisks) that are indicative of fiber type grouping were a common characteristic of aging in all mouse lines. Scale bar, 0.1 mm.

preference was more pronounced in rAg- and Ag-overexpressing animals. Together, these results indicated that disabling agrin cleavage at the two neurotrypsin-sensitive positions did not prevent sarcopenia. However, a beneficial influence of transgenic agrin is possible, in situations where neurotrypsin is not overexpressed. This beneficial influence could be observed in both resistant agrin and wild type agrin-overexpressing mice. Thus, it might be rather related to the amount of agrin than to the prevention of cleavage at the neurotrypsin-specific positions. This result does not generally exclude but also not support increased agrin degradation or decreased agrin synthesis as a cause of sarcopenia. Additional supply with agrin, however, could have beneficial influence on sarcopenia. Whether this is due to interference with fundamental sarcopenia promoting processes, or with regeneration or compensation mechanisms remains elusive.

4 DISCUSSION

We found that enhanced proteolytic cleavage of the NMJ stabilizer agrin, induced by transgenic overexpression of the neuronal serine protease neurotrypsin in motoneurons, results in reduced skeletal muscle mass and function with a sarcopenia-like histopathological phenotype. Characteristic alterations included a reduced number of muscle fibers, increased heterogeneity of fiber thickness, angular fibers, centralized nuclei, fiber type grouping, and an increased proportion of type I fibers (**Table VIII**). In previous studies, agrin has been identified as proteolytic target of neurotrypsin. As in age-dependent sarcopenia, the characteristic muscular alterations were accompanied by severe fragmentation of the NMJs. In the most advanced cases, highly fragmented presynaptic nerve terminals were found without opposed postsynaptic specialization. These structures may represent a final stage indicating imminent NMJ dispersal, as agrin-free and therefore unprotected postsynaptic specializations are dispersed by a postsynaptic mechanism activated by acetylcholine-dependent signals[57,58].

Induced proteolytic cleavage of NMJ agrin mimicks the neuromuscular phenotype of sarcopenia in all tested aspects. Thus, these mice exhibit an aging-like neuromuscular system, in a well-defined and otherwise healthy organism. The induction and expression of the sarcopenia phenotype at juvenile and young age by agrin cleavage-dependent NMJ degradation indicates a key role of the NMJ for the integrity of the neuromuscular system at advanced age and positions the impaired NMJ as an access point to the final common pathway controlling the pathogenic processes resulting in sarcopenia. Besides enhanced cleavage and inactivation of NMJ agrin, other mechanisms affecting NMJ integrity may adopt this pathway for their sarcopenia inducing activity, including prominent suspects for primary causes of sarcopenia, such as age-dependent death of motoneurons and a decreased capacity of aged motoneurons to innervate regenerated muscle fibers. Several studies in rats and humans indicated a loss of motoneurons with aging and suggested motoneuron death with associated muscle fiber denervation as a cause for sarcopenia[144-146]. However, quantitative considerations indicated that motoneuron loss may not fully account for the loss of muscle fibers[147]. Therefore, it has been hypothesized that, with aging, motoneurons become completely or partially disconnected from their target fibers, possibly by a degradative process at the NMJ[148]. In accordance with this view, our results exclude motoneuron death as a necessary condition for sarcopenia. Our quantifications showed normal numbers of motoneurons in both young adult neurotrypsin-overexpressing and aged wild type mice. Therefore, denervation attributable to loss of motoneurons cannot be made accountable for NMJ decay and sarcopenia,

Table VIII
Neuromuscular deteriorations associated with sarcopenia

	Nto1	aged
motor coordination impairments	+	+
loss of strength	+	+
loss of muscle fibers	++	+
type I fiber preference	+	++
fiber type grouping	++	+
heterogeneity of fiber thickness	++	+
centrally nucleated fibers	++	+
angular fibers	++	+
denervation of fibers	?	?
infiltration of fat and connective tissue	-	(+)
endplate fragmentation	++	+
loss of motoneurons	-	-

Previously described age-associated neuromuscular degenerations and their occurrence or severity in neurotrypsin transgenic and aged mice.
++ numerous, severe; + frequent, medium; (+) sometimes observed, mild; - not observed; ? not determined.

neither in our experimental model nor in aged mice.

Together, the neurotrypsin transgenic mice can serve as model for sarcopenia, simplifying research on sarcopenia dramatically. Improvement of our understanding of sarcopenia, essentially the underlying molecular mechanisms, is crucial to develop novel strategies to treat or counteract the age-dependent muscle wasting.

4.1 Neurotrypsin can cleave agrin and agrin cleavage is sustained in senescence

As reported previously, agrin is a substrate of neurotrypsin *in vitro*[119] and *in vivo*[121]. The transgenic overexpression of the active serine protease neurotrypsin in mice leads to an increase in the amount of the agrin-90. This fragment is specifically produced by neurotrypsin-dependent agrin processing, since it is completely absent in neurotrypsin deficient mice. In this study, several transgenic mouse lines overexpressing neurotrypsin were used. The overexpression of neurotrypsin was associated with an increased agrin cleavage in all mouse lines overexpressing active neurotrypsin. The amount of agrin fragment correlated with the neurotrypsin expression level, confirming that agrin is proteolytically processed by neurotrypsin *in vivo*. Importantly, the protein level of neurotrypsin determined not only the grade of agrin processing but also the severity of the histopathological skeletal muscle phenotype.

Neurotrypsin expression and agrin cleavage are sustained in senescence, although the levels are decreased. Thus, the cleavage of agrin may not only be important in early development but also during adulthood and age. Neurotrypsin expression and agrin cleavage have been shown to predominate in the first two weeks postnatally, and may be implicated in the development of the NMJ from plaques to pretzel. Agrin processing is thought to dissolve agrin-derived restraints on the NMJ, facilitating the spatially restricted dispersal of postsynaptic sites to reach the typical pretzel-like shape of the adult NMJ. During adulthood, the NMJ is rather stable, in agreement with decreased agrin cleavage. A basic level of agrin cleavage may ensure the plasticity of the NMJ during this period. Interestingly, the most heavily glycanated variants of agrin disappear in age. Whether this is mainly due to increased processing or decreased synthesis is not clear. Neurotrypsin is not or not alone responsible for the disappearance of these agrin variants, since they also disappear in Ntd mice, suggesting another neurotrypsin-independent agrin degrading process. Previously, it has been reported that the most heavily glycanated agrin variants predominate at synapses and are mainly cleaved by neurotrypsin in mouse brains[121]. Decreased amount of synaptic agrin, regardless whether due to increased processing or decreased synthesis, may substantially influence NMJ stability, and hence, the development of sarcopenia.

4.2 Neurotrypsin overexpression leads to a sarcopenia–like decline in skeletal muscle mass and function

Different transgenic mouse lines overexpressing neurotrypsin neuronally or motoneuronally exhibited a loss of muscle function, mass and strength, which strikingly resembles sarcopenia.

Movement disorders, such as incorrect paw placement, short stride length, and trembling and staggered movement are typical for transgenic and for aged mice, suggesting similar etiologies. Since motoneuronal neurotrypsin overexpression is sufficient to induce this motor coordination impairment, muscular or neuromuscular processes and not a central mechanism are involved. The motor coordination impairment was accompanied by a loss of strength in both neurotrypsin-overexpressing and aged mice. Consistent with this finding, muscle mass and thickness were

reduced. Loss of muscle mass and thickness has been shown to be the main reason for the loss of strength with aging. Multiple studies have shown a correlation between muscle mass and strength[177,179,193,194]. In agreement with this finding, reduction of skeletal muscle mass, strength and thickness all were of similar magnitude in transgenic mice (about 10 % in line Nto1). Whereas loss of strength could be explained by reduction in muscle thickness, the motor coordination impairment seen in transgenic and aged mice suggests more complex disturbances of the neuromuscular system.

The relatively moderate loss of muscle mass and CSA was accompanied by a dramatic loss of muscle fibers, which can account for more than 40 % of all fibers. The loss of fibers was by far more severe than the loss of muscle thickness and strength in neurotrypsin-overexpressing and in aged animals. This obvious discrepancy can be explained by a compensatory hypertrophy of a part of the remaining muscle fibers, which is responsible for an increased heterogeneity of muscle fiber thickness. Unlike in general muscle atrophy only a subset of muscle fibers is reduced in thickness, whereas others undergo a substantial expansion. The existence of hypertrophic and atrophic fibers within the same muscle has been reported to be an important characteristic for sarcopenic muscles[238]. The pathogenic mechanism underlying sarcopenia seems to distinguish between fibers within the same muscle, causing an increased variability of fiber thickness. Increased heterogeneity of fiber size in conditions with enhanced agrin cleavage provides evidence for similarities in the processes leading to age-related and agrin cleavage-induced sarcopenia. Healthy, unaffected fibers are hypertrophied to compensate the loss and atrophy of others, a process that can actually result in an increase in mean fiber thickness. This compensation process can be strikingly efficient: comparison of muscle mass per body mass revealed no change between wild type and neurotrypsin transgenic animals, despite a loss of more than 30% of the muscle fibers. With age, muscle mass and thickness not even dropped significantly, despite a dramatic loss of fibers. The age-related increase in body mass, increasing the load on the muscles, may further intensify the compensatory effect. Increased loading of a postural muscle, such as the soleus, can't be completely avoided by a decline in activity and may lead to a greater training effect. Furthermore, age-related increase in non-contractile muscle content, due to the infiltration of fat and connective tissue may also counteract the fiber loss-induced reduction in muscle thickness. Relative non-contractile muscle components have been shown to significantly increase with age in rats and humans[189,190,197,198,209,243,274-276]. This may be attributable to decreased physical activity, since increase in non-contractile muscle components have also been observed following hindlimb suspension in rats[508,513,514]. In agreement with these reports, we occasionally observed the infiltration of fat and connective tissue in the muscle of aged mice suffering from sarcopenia, but not in the muscles of transgenic mice, which may maintain normal physical activity.

The appearance of small angular fibers, which are considered to be evidence for denervation[158,197], suggests that the disconnection of motoneuron and myofiber might be the onset of muscle fiber atrophy and loss in transgenic and in aged animals. Denervation removes the trophic influence on the fiber and leads to atrophy[283,308,309]. Especially experimentally induced partial denervation resembles sarcopenia[329], suggesting that denervation of a subset of muscle fibers may be involved in the development of sarcopenia. Partial denervation can also explain the presence of atrophied potentially denervated fibers, among normal and hypertrophied fibers that obviously are still functionally innervated, within the same muscle.

Furthermore, centrally nucleated muscle fibers provide evidence for regeneration in both neurotrypsin-overexpressing and aged animals. The muscle tries to overcome the loss of fibers by adding new centrally nucleated fibers. An age-related increase in centrally nucleated fibers has previously been found in several studies[242,271]. However, this regeneration is inefficient, since finally the loss of fibers can't be stopped. Speculations about the reasons for incomplete regeneration in

senescence involve impaired proliferation[284,285], activation[292], or differentiation[290] of satellite cells as well as incomplete innervation of regenerated fibers[147]. The presence of sarcopenia in young adult animals, which are not likely to suffer from age-related changes in satellite cell metabolism, strongly promotes incomplete innervation, possibly due to failure in NMJ formation, as reason for unsuccessful regeneration. Moreover, impairment in satellite cell metabolism would eventually also influence the ability of muscle fibers to get hypertrophied. Satellite cell recruitment and fusion appears to be requisite for load-mediated hypertrophy[302-304,464]. There is a causal link between satellite cell proliferation, increased myonuclear number, and fiber hypertrophy. The myonuclear domain, the amount of cytoplasm an individual nucleus is controlling, does not or only marginally change in response to overloading-induced hypertrophy. Therefore, the substantial skeletal muscle hypertrophy seen in neurotrypsin-overexpressing and in aged animals may exclude a general failure of satellite cell metabolism.

4.3 Dose dependency and rescue experiments emphasize enhanced agrin cleavage as essential step to neurotrypsin-induced sarcopenia

The severity of the sarcopenia-like muscle wasting was in good correlation to the level of agrin cleavage in line Nto1 and Nto2. Enhancement of agrin cleavage, represented by increased amounts of agrin-90, leads to more extensive fiber loss, increased heterogeneity of fiber size, and more centrally nucleated and angular muscle fibers. This provides good evidence that enhanced neurotrypsin-dependent agrin cleavage causes the muscle wasting. Importantly, motoneuronal neurotrypsin expression is sufficient to induce the whole phenotype. It is presynaptically secreted and may cleave agrin at the basal lamina of the NMJ. In agreement with the importance of motoneuronal neurotrypsin expression, the Nto-moto mouse line showed a very severe phenotype, although the overall neurotrypsin expression and agrin cleavage in the spinal cord were at a similar level as in the weaker line Nto2. The expression level in motoneurons may actually be higher in Nto-moto than in Nto2 animals, where neurotrypsin is distributed over all neurons. Higher level of motoneuronal neurotrypsin expression may induce a more severe phenotype.

Mice overexpressing an inactive form of neurotrypsin can't be distinguished from wild type littermates in any respect, indicating that the proteolytic activity of neurotrypsin is responsible for the degenerative changes. Furthermore, the concomitant overexpression of neurotrypsin and a neurotrypsin resistant agrin form completely rescued from the muscle wasting phenotype. This indicates that the excessive cleavage of agrin by neurotrypsin is the important step towards neurotrypsin-induced sarcopenia. The cleavability of agrin at the neurotrypsin-specific α and β position is essential to precociously induce sarcopenia. All the described morphological alterations following neurotrypsin overexpression and excessive agrin cleavage can similarly be observed in aged mice. Interestingly the relative strength of the different histopathological alterations is similar in conditions with enhanced agrin cleavage and in senescence. Generally, all effects in aged animals are less severe than in Nto1, but more severe than in Nto2 mice. The presence of the whole ensemble of sarcopenic changes in young transgenic mice indicates that the pathogenic mechanism underlying agrin cleavage- and aging induced sarcopenia may be similar.

4.4 Muscle specific differences in neurotrypsin-induced sarcopenia

To confirm that neurotrypsin-induced sarcopenia does not only affect the soleus muscle, a second muscle exhibiting completely different characteristics has been analyzed. The EDL is a fast-twitch muscle, which is mainly composed of type II fibers, in contrast to the slow-twitch soleus muscle.

Moreover, the EDL is a locomotor muscle, which is important for the movement of the animal, whereas the soleus is a postural muscle that is also activated when the animals does not move. In conditions with enhanced agrin cleavage, muscle mass and fiber number of both soleus and EDL muscle are reduced. However, the soleus muscle showed more pronounced decline in both mass and fiber number. Interestingly, considerable muscle specific differences can also be found in regard to the endplate fragmentation following excessive agrin cleavage. Especially sternomastoid and diaphragm muscle have been analyzed in this respect in our lab. Slow-twitch diaphragm showed very severe NMJ degeneration with a complete disappearance of normal endplates, whereas in sternomastoid muscle the NMJs were rather resistant against neurotrypsin-induced fragmentation. A low stability of slow, type I fibers or endplates could explain the deleterious effects on the slow-twitch muscles soleus and diaphragm. However, following neurotrypsin overexpression the soleus muscle becomes increasingly slow, indicating a higher stability of type I and not type II fibers. Alternatively and more plausible, the expression level of neurotrypsin in the motoneurons innervating distinct muscles could be different. We have shown that the expression level of neurotrypsin has a direct influence on the extent of agrin cleavage and the severity of the sarcopenia phenotype. Expression of neurotrypsin is controlled from the Thy1 promotor that could be differentially regulated in motoneurons innervating distinct muscles.
In aged animals, both soleus and EDL are similarly affected in regard to fiber number. Muscle mass, however, is more reduced in EDL muscle, which could be attributable to more extensive type II fiber atrophy. Selective reduction in type II fiber thickness has been found in numerous studies on aged rodents and humans[232,239-245]. Alternatively, compensatory hypertrophy might be more pronounced in a postural muscle, such as the soleus, than in a locomotor muscle, such as the EDL, due to a greater training effect. The use of a locomotor muscle might be reduced in age, as reduced physical activity is a common and often observed consequence of aging. However, decreased physical activity does not reduce loading on a postural muscle to the same extent. Therefore, compensatory hypertrophy may be more pronounced in soleus than in EDL muscle.

4.5 Fiber number estimations from cross-sections

There has been an extensive discussion about the problems of using histological sections for determination of fiber number[522,523,644]. The accuracy of the enumerations crucially depends upon whether or not a cross-section includes all the muscles fibers, and this in turn depends on the structure of the particular muscle. Especially for muscles with pennate fiber arrangements, wrong estimates of the fiber number form cross-sections can be made. However, in muscles with parallel fiber arrangements, such as the soleus and the EDL, this method of fiber enumeration appears to be feasible, as long as the muscle is cut at right angle to its long axis. Timson et al.[645] used histological sections as well as the nitric acid digestion method[522,646] to estimate the number of fibers in the soleus muscle of mice. Both methods gave similar results, confirming that fiber counting on cross-sections can be used to determine the soleus fiber number. Nevertheless, it is important to use sections from the mid-belly region of the muscle, because the number of fibers appearing in a histological section decreases from the belly to the distal end of the muscle. That is because the fibers of the soleus muscle do not run the entire length of the muscle. Consequently, the mean fiber length is only about 60-70% of the muscle length. In the present study, cross-sections with an interspace of less than 150 μm covering the whole mid-belly region of the soleus muscle were prepared. The fibers appearing in the sections were counted, and only the two highest numbers were used for the fiber number estimates of the muscle. With this method, influences of the position of the cross-sections in the muscle can be minimized.
The soleus muscle of mice has been previously studied in regard to fiber number, area and

composition[645]. The soleus was found to be composed of about 950 fibers, regardless of the sex of the animals and the method of fiber enumeration. This value is very similar to the results in the present study. The fiber area was slightly smaller than our results, which could be attributed to different mouse strains and ages (BALB/c, 8-7 month old instead of C57Bl/6, 4 month old). The percentage of type I fibers was reported to be 57% and 46% for females and males, respectively. We found 40% and 30% of type I fibers for female and males, respectively. These variations are not surprising since it has been shown that the fiber type composition can vary considerably among different rodent strains[200]. Vaughan et al.[258] determined the fiber type composition of the soleus muscle in two different mouse strains and found considerable differences. The percentage of alkali-labile type I fibers was 45.7% and 59.7% for males and females, respectively, in strain Hul129Re and 58.4% and 65.8% in strain HulT0. Genetic differences among different stocks of mice have been thought to influence the fiber type composition and could well be responsible for the lower percentage of type I fibers in our study. Furthermore, Timson et al.[645] and Vaughan et al.[258] used myofibrillar ATPase staining following preincubation at pH 10.3 and 10.4, respectively. This results in different staining intensities among the different type II fiber types and leaves the alkali-labile type I fibers unstained[229]. This method bears the risk of classifying some of the weakly stained type IIb fibers as non-stained type I fiber, leading to an overestimation of type I fiber numbers. In the present study we used mATPase staining following preincubation at pH 4.3, which only and strongly labels the acid-stable type I fibers. In this way, we could exclude the risk of wrong classification. Significant sex differences in regard to fiber type composition of the soleus muscle have been found in all studies. Vaughan et al.[258] speculated about an influence of androgens on the percentage of type I fibers since this percentage was similar between castrate males and females. Interestingly, even castration at an age of 4 month, at an age by which the "male" pattern of fiber types should have been fully established, resulted in a significant increase in type I fibers. It therefore appeared that continuously high androgen levels are required to maintain the low proportion of alkali-labile type I fibers. Changes in fiber type composition can be established without interfering with innervation, which is generally thought to determine the fiber type[230].

4.6 The whole sarcopenic phenotype is installed in a few weeks after the onset of neurotrypsin overexpression

A developmental study of the muscle fiber number showed, that the onset of the fiber loss was slightly before postnatal day 10 (P10). The onset of neurotrypsin overexpression has been determined to be around P6[634] (Bolliger et al., in preparation). It takes only a few days until excessive agrin cleavage and destabilization of the NMJs results in loss of muscle fibers. Interestingly, most of the fibers are lost in the first month postnatally. Later, the number of fibers in transgenic animals stayed constant, although the overexpression of neurotrypsin was maintained (**Fig. 7c**). One possibility could be that neurotrypsin overexpression and excessive agrin cleavage interferes only with initial stages of muscle growth. Once the muscle fiber has been established, it is stable. However, a drop in the number of fibers during the third and fourth postnatal weeks indicates that already formed fibers are actively lost in transgenic animals. Alternatively, muscle fibers are constantly lost but regeneration could compensate for degeneration in adult mice, building a dynamic equilibrium. Morphological indications for regeneration, such as central nuclei and axonal sprouting, support this hypothesis. Regeneration might be delayed in respect to the start of the fiber loss, but could then be responsible to maintain a dynamic equilibrium. Nevertheless, during initial growth of the muscle, the action of neurotrypsin seems to be especially fatal. A few days after onset of neurotrypsin overexpression, muscle fibers are lost and some weeks later the whole sarcopenic phenotype is installed.

Fiber hypertrophy can be observed in the transgenic animals after day 45 postnatally, when the growth of the muscle is about to be concluded. Since fiber hypertrophy is thought to compensate for atrophied or lost fibers it is not surprising that it is delayed in regard to the start of the fiber loss. After maturation, growth of individual muscle fibers in transgenic muscles continuous, probably as a reaction to increased loading after atrophy and loss of a subset of fibers.
In wild type animals, the muscle fiber number was found to increase during the first two to three weeks postnatally. Thereafter it remained constant during adulthood. Whether postnatal increase in fiber number actually occurs, and if it does, in which time scale and to which extent has been conversely discussed in the literature. Whereas some studies did not find any increase in the number of myofibers after birth[647,648], others showed a substantial increase in the first postnatal weeks[649-651]. Rayne and Crawford[644] analyzed the medial and lateral pterygoid muscle of rats at birth, and one and six weeks after birth. The number of fibers in the medial pterygoid muscle almost doubled in the first week after birth, and between first and sixth weeks there was a further small increase of about 20%. In the lateral pterygoid muscle, the postnatal rate of increase of fiber number was 12 and 20% between newborn and one week old, and between one and six weeks old animals, respectively. Recently, it has been found that the there is no net gain in myofiber number of mouse EDL muscle between P7 and P56[652]. Myofiber CSA, in contrast increased more than 7-fold in the same time period. Together, most of the muscle fibers appear to be present at birth, although some increase in fiber number might occur during the first weeks postnatally. This may be attributable to the differentiation of myoblasts present in muscle fiber bundles at birth into myofibers. Not fully differentiated muscle fibers are not always included into the counting, leading to different results. However, it is well established that the main growth of the muscle after birth is due to expansion of the existing muscle fibers and that the number of fibers stays constant during adulthood. In agreement with these studies, we found a small increase in fiber number in the first two postnatal weeks that is accompanied by a large increase in fiber thickness. Increase in muscle CSA appears to be concluded around day 45, a finding that correlates with findings from earlier reports[647].

4.7 Deterioration of the NMJ is responsible for agrin cleavage- and aging-induced muscle wasting

4.7.1 Excessive agrin cleavage and aging results in fragmentation and dispersal of NMJs

Analysis of the NMJs of neurotrypsin transgenic and aged animals supports the hypothesis that denervation of a subset of muscle fibers causes both agrin cleavage- and aging-induced sarcopenia. A higher proportion of fragmented NMJs may be an indication for the ongoing deterioration of the neuromuscular connections. Fragmentation included the replacement of the typical pretzel-like shape by numerous small spots of pre- and postsynaptic specializations. In the majority of cases, the alignment of pre- and postsynaptic sites was maintained. However, partial misalignment between highly fragmented presynaptic nerve terminals and corresponding postsynaptic sites occurred. Moreover, presynapses that were opposed by a cloudy, very weak postsynaptic signal and, finally, presynapses that were not opposed by postsynaptic AChR clusters can occasionally be observed in conditions with excessive agrin cleavage and with aging. These structures could represent final stages on the way to the disappearance of the NMJ. Synaptic transmission is not likely to occur, resulting in a functional denervation. Importantly, these structures suggest, that the dispersal of the postsynaptic sites precede the degeneration of presynaptic nerve endings.
The morphology of the NMJ has been shown to depend on the type of the corresponding muscle fiber[653]. Total number of nerve terminals and endplate branches increases progressively from type I, IIa, IIx to IIb fibers. Therefore, fiber type changes could have an influence on the amount of

fragments per endplate. We observed an increase in proportion of type I fibers upon both enhanced agrin cleavage and aging. More type I endplates with a low number of branches would lead to a decrease in the number of fragments. However, the opposite, an increase in the number of fragments, was observed. Thus, a causal role of fiber type changes on endplate fragmentation can be excluded in both neurotrypsin overexpression and aged animals.

It is well-established that agrin is crucial for the maintenance of the NMJ[13,26]. The cleavage of agrin by neurotrypsin removes the C-terminal fragment, containing the AChR clustering activity of agrin, which is essential for the NMJ stabilizing function of agrin. Agrin cleavage is therefore likely to destabilize the NMJ, by removing the protective function of agrin. It has been shown that unprotected, agrin-free postsynaptic specializations were dispersed during postsynaptic differentiation in an acetylcholine dependent manner[57,58]. Consistent with this idea, silencing the expression of MuSK, the downstream target of agrin, leads to the disassembly of existing NMJs[91]. This process included fragmentation and severe disassembly of postsynaptic AChR clusters. In response to abrogation of postsynapse integrity, presynaptic nerve endings began to sprout. In some severe cases, the entire postsynaptic structure was lost and only the motor nerve terminal was remaining. Thus, presence of MuSK and agrin are required for ongoing maintenance of the NMJ. A similar mechanism may be responsible for the dispersal of the NMJs after excessive cleavage and hence, inactivation of agrin. Such a mechanism would also explain the sequence of events. The excessive agrin cleavage at the NMJ removes the protective function of agrin. Acetylcholine, which is released by the nerve terminals induce the dispersal of the unprotected postsynaptic sites. This event may start at a certain site at the NMJ, leading to partially misaligned structure, and subsequently proceed over the whole NMJ. The dispersal may occur by internalization as well as by distribution of AChR in the muscle membrane. The cloudy, weak signals seen for AChRs in conditions with enhanced agrin cleavage could represent internalized and low concentrated receptors. At a certain stage, the amount of receptors may be too low to be detected. The dispersal of the postsynaptic sites may then lead to the retraction of the presynapse. It has been shown that nerve terminals are dependent on ongoing production of trophic factors by the postsynaptic muscle fiber[60]. Dispersal of postsynaptic sites and hence, shutdown of trophic factor supply is likely to result in the retraction of the nerve terminal. If the retraction of the nerve would precede the dispersal of postsynaptic sites, non-occupied receptor cluster would be visible, because the postsynaptic apparatus can persist for a long time in the absence of a nerve[84-86]. The absence of agrin-mediated stabilization as well as the presence of ACh-mediated destabilization is needed for active dispersal of postsynaptic AChR clusters. Absence of the nerve leads to both absence of agrin and ACh, resulting in relatively stable postsynaptic AChR clusters. Such structures have not been observed in conditions with enhanced agrin cleavage, strongly suggesting that the dispersal of the postsynaptic site precedes and induces the dispersal of the presynaptic site. Whether excessive agrin cleavage is also responsible for the NMJ fragmentation and the muscle wasting observed with aging is presently unknown. However, the mechanism of agrin cleavage- and aging-induced neuromuscular degeneration seems to be strongly related. With aging, as with increased agrin cleavage, NMJ fragmentation of both pre- and postsynaptic sites in a concerned fashion rather suggests active dispersal of postsynaptic sites than unilateral deterioration of the nerve ending. Deterioration of nerve terminals would result in unoccupied AChR clusters, which persist due to the absence of nerve-derived dispersal. Such structures were not observed in aged animals. Interestingly, in response to nerve injury, aged NMJ exhibited significant fragmentation and loss of motor endplate area while the young NMJ remained relatively stable[343]. Similarly, following muscle unloading, aged NMJs undergo significant remodeling of both pre- and postsynaptic parts, whereas adult NMJs maintain normal morphological characteristics[405]. Aged endplates appear to be more sensitive to the stimulus of unloading, possibly due to a general increase in plasticity that

is accompanied by a decrease in stability. It's possible that lower amounts of full-length agrin are responsible for the destabilization of aged NMJs. Both increased agrin cleavage and decreased agrin synthesis would influence the amount of full-length agrin capable of stabilizing NMJs.

4.7.2 Endplate fragmentation is accompanied by a loss of synaptic area

The fragmentation is associated with a loss of synaptic area in transgenic animals. Although muscle thickness has been shown to greatly influence synaptic area[82,83], this effect can't be attributed to a decrease in muscle thickness, since mean muscle fiber thickness is actually increased in these animals. The fragmentation does not only include a dispersal of the existing pre- and postsynaptic sites into small spots, but also an active loss of these sites. Interestingly, the synaptic area was increased in Nto1/rAg animals compared to wild-type littermates. In concert with this finding, the NMJs of these animals exhibited a more compact shape with a reduced proportion of AChR-free area. Some of these endplates rather resembled large plaques with some perforations than mature pretzel-like structures. Previously, it has been shown that the maturation of endplates during postnatal development is delayed in mice concomitantly overexpressing neurotrypsin and resistant agrin (Bolliger et al., in preparation). This finding has been attributed to difficulties in resolving the agrin-induced restraints during maturation, since agrin can't be cleaved by neurotrypsin. Nevertheless, most of the endplates reaches the pretzel stadium. However, it is plausible that the shape of these NMJs remain to be more compact in adulthood, because the generation of AChR free areas, which relies on the removal of agrin's protective function by its cleavage, is impeded. In aged animals, a significant decrease in synaptic area was not detected. This is not surprising, since the fragmentation was clearly less pronounced in aged than in transgenic animals. So far, a number of studies tried to analyze age-related alterations in the size of both pre- and postsynaptic parts of the NMJ[93,343,401,402,405,406]. However, consistent results were not obtained. High natural variations, influences of muscle and fiber type on NMJ size, and methodological difficulties complicate the analysis of age-related changes of synaptic area and may explain the variety of results obtained. Our data does not indicate an age-related decrease in endplate area in the age range analyzed. However, the transgenic situation implies that when the fragmentation eventually gets more severe at higher age, this could be accompanied by a loss of synaptic area.

4.7.3 Agrin cleavage appears to be increased at fragmented NMJs

Concomitant staining of NMJs for the two C-terminal cleavage fragments that result from neurotrypsin-dependent agrin processing provided insights into the dynamics of agrin-mediated NMJ stabilization. The agrin middle fragment, agrin-90, remains associated to fragmented NMJs, whereas the most C-terminal fragment, agrin-22, seems to disappear after cleavage. Signals for agrin-22 were clearly weaker in fragmented NMJs of neurotrypsin-overexpressing animals, whereas the signal intensity for agrin-90 appears not to change greatly. In some cases, agrin-22 may be completely absent. These finding suggest that neurotrypsin-dependent agrin processing at the corresponding α- and β- cleavage sites results in the disappearance of agrin-22, whereas agrin-90 remains associated with the NMJ. The small C-terminal fragment, which contains the AChR clustering activity, is likely to be diffusible, because it can be found in the cerebral spinal fluid and in the blood of mice and humans (unpublished observation). Agrin-90, in contrast, contains binding sites for α-dystroglycan, heparin, and integrins[99] and might be trapped at the basal lamina of the synaptic cleft after cleavage. In contrast to full-length agrin, agrin-90 is not capable of inducing and protecting postsynaptic AChR clusters, indicating that the cleavage of agrin might be responsible for a loss in agrin-mediated NMJ stabilization. Further evidence for this hypothesis is

provided from the observation that agrin-22 and AChR aggregates concomitantly disappear from heavily fragmented NMJs. The absence of agrin-22, indicating enhanced agrin cleavage, prevents agrin signaling and hence, the stabilization of the postsynaptic AChR clusters. Accordingly, the absence of postsynaptic AChRs, leading to abandoned nerve terminals, was always accompanied by the absence of agrin-22. The absence of agrin-22, on the other hand, not always co-occurred with the absence of AChR aggregates, indicating that agrin processing and diffusion of agrin-22 might precede the dispersal of postsynaptic sites. The question about the fate of agrin-90 after agrin cleavage can't be answered conclusively. Agrin-90 always remained associated with the NMJ, even in abandoned nerve terminals lacking the postsynaptic sites. Since agrin-90 may not be diffusible in the basal lamina of the synaptic cleft, internalization is a plausible scenario. The signal at abandoned nerve terminal could represent agrin-90 that has not yet been internalized. Since such nerve terminals may not represent stable and long-living structures, it is plausible that agrin-90 is not internalized during the lifetime of these structures. Alternatively, it's possible that a part of the signal could represent internalized agrin that lingers in vesicles beneath the NMJ.

4.7.4 Evidence for denervation- and reinnervation events

The innervation of highly fragmented endplates remains largely intact. Therefore, degeneration of motoneurons can be excluded as cause for endplate fragmentation and dispersal. Moreover, the motoneurons still have the potential the innervate muscle fibers in neurotrypsin transgenic animals. Bolliger et al. (in preparation) found striking outgrowth of nerve fibers on the diaphragm muscle of neurotrypsin transgenic mice (**Fig. 2c**). The nerves were not capable of building stable NMJs and grew over the muscle surface. The formation of small, unstable synapses has been observed. Unfortunately, the outgrowth of nerves can't be observed on the soleus muscle, because it has to be teased into small bundles of fibers to stain and image the NMJs. During this process the initial positions of muscle fibers to each other is lost. However, very small immature synapses could represent attempts of outgrowing motoneurons the form new synapses. Moreover, terminal sprouting of nerves from endplates were observed on the soleus muscle of neurotrypsin transgenic mice. Terminal sprouting is known to be induced by adjacent, inactive muscle fibers, since presynaptic blockade with botulinum toxin, nerve conduction block with tetrodotoxin and spinalization have been shown to induce terminal sprouting[331]. Fragmentation and dispersal of NMJs following enhanced agrin cleavage may lead to a loss of connection between nerve and muscle and, subsequently, to inactivity of the muscle fiber. Inactivity of the fiber induces sprouting of adjacent nerves to reinnervate the denervated fiber, a process that has been observed in conditions with enhanced agrin cleavage and that finally leads to fiber type grouping. Terminal sprouting has also been reported to substantially increase with age[336,410], adding another line of evidence for a common pathway leading to neurotrypsin-induced and aging-induced muscle weakness.

4.7.5 Deterioration of the NMJs influences muscle morphology

In neurotrypsin transgenic and in aged mice, completely normal, as well as highly fragmented NMJs can be found. This pattern influences the muscle morphology and could be responsible for increased heterogeneity of fiber thickness. Muscle fibers with highly fragmented or dispersed NMJs loose the contact to their motoneurons and start to shrink, being angular and eventually degenerate. Subsequently, the loading on normally innervated fibers with healthy fully functional synapses increases. These fibers start to become hypertrophied and compensate, at least partially, for the loss and atrophy of fibers. This process is likely to be dynamic; healthy synapses may also start to get fragmented.

The muscle itself exhibits signs of regeneration, trying to overcome the loss of muscle fibers by adding new ones. However, regeneration is inefficient, probably due to difficulties in establishing stable and functional NMJs. The ability of motoneurons to form stable NMJs influences the efficiency of reinnervation of denervated or newly added muscle fibers and hence, the efficiency of regeneration. Without agrin's synapse protecting function, it might be possible to build transient, unstable synapses, but not a fully functional endplate. Small immature spots with pre- and postsynaptic labeling could represent the attempts of motoneurons to induce new synaptic sites, without actually being successful. Similarly, terminal sprouts might not be able to reinnervate adjacent, denervated muscle fibers due to the inability to establish stable NMJs. Loss of muscle fibers in concert with incomplete compensatory hypertrophy leads to reduced muscle size and mass, which is responsible for the decline in muscle strength. The accumulation of partially or fully denervated, reinnervated, atrophied and hypertrophied or newly added muscle fibers, could be the reason for the coordinative problems of the mice. It could be difficult to control an unstable, de- and regenerating neuromuscular system.
Interestingly, it has been shown that aged motoneurons appear to be less successful than adult motoneurons in innervating denervated muscles[147]. As in conditions with enhanced agrin cleavage, the inability to form stable NMJs might be the reason. Aged motoneurons may not be capable to establish a stable and enduring contact to the muscle. Fragmentation of the NMJ may also be a possible explanation for the differences in age-related loss of muscle mass and function. Muscle fibers that have lost their motoneuronal input can't be activated but may still be present for a certain time. They contribute to muscle mass but not to muscle function. Such a process would lead to a discrepancy between loss of muscle mass and function, which has been observed with aging[180,183,186,194,206,207]. However, in a human study, age-related decline in specific force has been detected, despite the old subjects were able to fully activate their muscles[210]. It would be interesting to analyze specific force in neurotrypsin-overexpressing animals to understand to which extent NMJ deterioration can influence specific strength. So far, there is no evidence for loss of specific strength, since loss of soleus and EDL muscle mass and grip strength were of similar magnitude. However, precise analysis, including the measurement of strength and size of the same muscle, would be needed to address this issue.

4.8 Denervated muscle fibers were not detected in transgenic mice

The appearance of angular muscle fibers, nerve terminals that do not contact muscle fibers and terminal sprouts provide indirect evidence for the denervation of muscle fibers in transgenic and aged mice. Several methods to directly detect denervated muscle fibers in the soleus muscle were applied. However, a clear result could not be retrieved, although a tendency for an increased proportion of denervated fibers was observed. Three reasons may be responsible for the imperfect results. First, the methods were not sensitive enough or not correctly executed, second, denervated muscle fibers are quickly reinnervated or degraded, or third, denervated myofibers do rarely appear in transgenic or aged animals.
We used different markers for denervated muscle fibers, but all of them were not completely feasible. Two of them had been previously detected in totally denervated muscles (AcPH3, myogenin)[643] and may be difficult to use in a partially or only slightly denervated muscle. The detection of the $Na_v1.5$ sodium channel was not possible, since the commercially available antibody did not to work for immunostainings. NCAM was most promising, but the detected differences were small in contrast to the variation in a muscle and the possibility of false positives due to the preparation. Together, it would be crucial to have adequate controls. A muscle, which is completely denervated and a muscle, which is completely innervated should be available.

The amount of detectable denervated fibers depends on the time a denervated fiber stays denervated. If such fibers are quickly reinnervated or degenerated, their amount could be low, even when denervation frequently occurs. The detection would be difficult, especially, when the methods are not very sensitive. Nevertheless, an increased proportion of denervated fibers has been reported for aged rats and mice using NCAM antibodies[208,257] or a combination of electrophysiological and immunohistochemical methods to detect tetrodoxin (TTX)-resistant sodium channels[330], respectively. However, the proportion of denervated fibers varied substantially (8% and 4% vs. almost 50%), indicating methodological difficulties.
Interestingly, a 4-fold increase of the signal for myogenin has been found in western blots from gastrocnemius muscle extracts from aged compared to adult animals. Myogenin is involved in several aspects of skeletal muscle fiber development and repair. It has been found to be upregulated in denervated[607,608] and in regenerating muscles[604-606]. Transcription of myogenin is repressed by electrical activity and consequently, blockade of muscle electrical activity by denervation induces the re-expression of myogenin[609]. Therefore, age-related upregulation of myogenin could indicate the appearance of denervated muscle fibers. Myogenin expression increased almost 20 fold following complete denervation of the tibialis anterior muscle of the mouse[643]. However, myogenin mRNA increased also 18-fold due to regeneration following bupivacaine injection, which causes a dissolution of the sarcolemma that leads to rapid muscle fiber necrosis and subsequent recapitulation of myogenesis[610]. Thus, myogenin upregulation does not necessarily indicate denervated fibers but could also indicate ongoing muscle regeneration. The 10 fold upregulated of mygenin mRNA in tibialis anterior muscle of aged rats has been attributed to a continued attempt to generate new muscle fibers and ameliorate muscle atrophy. Surprisingly, in contrast to the results in aged animals, increase in myogenin was not constantly observed in conditions with enhanced agrin cleavage, despite a strong regenerative phenotype. However, myogenin upregulation is very fast and reversible. Myogenin mRNA expression was for example strongly increased 5 days after bupivacaine injection into the tibialis anterior muscle of adult rats. Already 14 days after induction of necrosis and subsequent regeneration, myogenin mRNA is downregulated to almost baseline levels[610]. The measured myogenin level is just a snapshot and thus, might not necessarily represent the continuous grade of denervation and regeneration. Moreover, it is not known, how myogenin is regulated after prolonged time of degeneration and regeneration in adult animals, as it is the case in neurotrypsin-overexpressing mice. Myogenin might not be directly associated with the degree of regeneration and denervation, at least not under conditions were these processes continues over a prolonged time period.
Together, we couldn't find direct evidence for the appearance of denervated muscle fibers in conditions with enhanced agrin cleavage or with aging, mostly due to methodological problems. Therefore, the performed experiments do not exclude, but also not confirm the denervation of muscle fibers as a step in the pathogenic process leading to sarcopenia. However, there is striking indirect evidence for denervation of muscle fibers in neurotrypsin transgenic and aged muscles, such as the appearance of angular muscle fibers, abandoned nerve terminals, and terminal sprouts.

4.9 The number of motoneurons is not affected by neurotrypsin overexpression or aging

A loss of motoneurons is not likely to cause the disassembly of both, pre- and postsynaptic sites, because the postsynaptic apparatus can be maintained in the absence of a nerve. Moreover, the innervation of fragmented endplates remains intact, indicating that events at the NMJs themselves and not motoneuronal loss induce NMJ fragmentation and dispersal. In agreement with these

findings, a loss of motoneurons has not been observed in transgenic animals. Endplate fragmentation and muscle wasting is not preceded or accompanied by a loss of motoneurons. The decay of the endplates is not caused by a retrieval or loss of the motoneurons and does also not primarily result in a loss of motoneurons. This supports our hypothesis, that the cleavage of agrin destabilizes the NMJ. The motoneuron itself seems not to be affected and could well have the potential to innervate new muscle fibers. The formation of immature synaptic sites, fiber type switching, and fiber type grouping suggest that innervation could be successful at the beginning. However, the ineffective regeneration of the muscle indicates that a stable connection may not be formed.
In aged animals, a loss of motoneurons has also not been observed. This finding is contradictory to several studies, in which the loss of motoneurons has been associated with aging in humans[145,332,352] and rats[234]. However, our result is not surprising, taken the relative moderate loss of muscle fibers in aged mice compared to rats or humans and recent evidence that the loss of motoneurons does not fully account for the loss of muscle fibers[395]. Since few motoneurons are lost during normal aging, it has been hypothesized that motoneurons become completely or partially disconnected from the target fibers[147]. This hypothesis is supported by our data, which indicates a dispersal of the NMJ, leading to a loss of the connection between motoneuron and myofiber. So far, it was generally believed, that a loss of motoneurons is the start and the cause of age associated muscle loss[654]. The finding that the muscle loss is not accompanied by a similar loss of motoneurons, but a severe fragmentation of the NMJ, indicates, that the decay of the endplate could be the factor triggering sarcopenia. It has been suggested, that sarcopenia to a significant extent depend on a decreased capacity among motoneurons to innervate regenerating fibers[147]. The reason for the impaired innervating capacity of the motoneurons could well be the inability to build stable NMJs, which crucially depend on agrin.

4.10 Fiber type grouping indicates neuropathological processes following enhanced agrin cleavage and aging

Degradative processes at the NMJs may also be key to understand the fiber type redistribution towards an increased proportion of type-I fibers consistently found in sarcopenia. The NMJs of type I and II fibers exhibit different morphologies and therefore stabilities. Consistent with a role of NMJ degradation in sarcopenia, it has been found that the larger and less compact NMJs of type II fibers are more prone to fragmentation with aging[402]. Our fiber type assessment upon enhanced agrin cleavage in neurotrypsin-overexpressing mice also indicated an increased proportion of type I fibers. Since the absolute numbers were decreased for both type I and type II fibers, this result is qualitatively sufficiently explained by a preferential loss of type II fibers. However, as in aged humans with sarcopenia, our mice with enhanced agrin cleavage at the NMJ showed pronounced fiber type grouping. Type I fiber grouping occurs for example when denervated type II fibers are reinnervated by collaterals of the motor nerve of an adjacent type I fiber. Therefore, a relatively small difference in the percentage of lost type I and type II fibers may be accentuated by reinnervation and reprogramming of type II fibers by the motor nerves of the less affected type I fibers. Furthermore, type I motoneurons may have a higher potential to build a stable endplate. Together, a small difference in NMJ stability could lead to dramatic effects on the fiber type ratio. However, grouping of type I and type II fibers was observed, indicating that both type I and II motoneurons have the potential to reinnervate neighboring fibers. Fiber type grouping is thought to result from a continuous neurogenic disorder, since the motoneuron determines the type of the innervated fiber. So far, fiber type grouping has always been thought to indicate motoneuronal death. Death of a motoneuron leads to the denervation of the associated muscle fibers and opens the possibility for their reinnervation and reprogramming by sprouts from adjacent nerves. Our

findings demonstrate that the dispersal of NMJs can lead to extensive fiber type grouping without actually affecting the number or viability of motoneurons. Therefore, fiber type grouping is not necessarily associated with motoneuronal death.

An interesting observation was the difference in the relative type I fiber increase in female animals overexpressing neurotrypsin alone and neurotrypsin together with wild-type agrin, respectively. The motoneurons additionally overexpressing agrin could have a higher potential to build at least transiently stable endplates, due to the additional supply with transgenic agrin. Thus, their potential to takeover denervated fibers and to induce fiber type changes might be higher, resulting in a higher frequency of fiber type changes in the double transgenic animals. Finally, this process does not lead to an increased number of muscle fibers, since the newly formed connection may not be stable. Agrin appears to have an essential role controlling the reinnervation of muscle fibers. The capability of a motoneuron to innervate a muscle fiber could be determined by its agrin content.

A higher stability of type I endplates nicely explains the type I fiber preference in transgenic animals, but not the opposite effect in males from line Nto1. A negative insertion effect of the transgene is not likely, because the rescue experiment with the resistant agrin is working, indicating that the decrease in type I fibers is caused by excessive agrin cleavage. Also here, small differences at the beginning, could lead to a big alteration at the end. If initially type II motoneurons would, for example, have some small advantages, takeover events could result in a severe increase in the proportion of type II fibers. A possibility would be a lower neurotrypsin expression level in type II motoneurons, increasing their potential to build and maintain endplates. The reason for a lower expression level could be the insertion of the transgene in a region with a generally lower expression level in type II than in type I motoneurons. Such a region could be differentially regulated in females, not causing the same effect.

Together, the increase in proportion of type I fiber for the majority of lines could be explained by small differences in NMJ stability, which leads to the predominant denervation, atrophy and loss of type II fibers. Additionally, takeover of denervated type II fibers by type I motoneurons, which have a higher potential to build stable endplates, could further increase the proportion of type I fibers and lead to fiber type grouping. The additional supply with transgenic cleavable agrin may enhance takeover events and further increase the proportion of type I fibers without improving the overall muscle morphology. The opposite effect in males from line Nto1 seems to be an exception, whose reasons remain elusive.

Fiber type grouping[146,198,241,267] and, at least for rodents, increase in proportion of type I fibers[146,201,234,235,252,253,255,256] has been reported to be characteristic for sarcopenia. Indeed, we found a significant increase in type I fibers and fiber type grouping in aged animals. Speculations about the mechanism of age-associated increase in type I fiber proportion include greater stability of type I muscle fibers, motoneurons, or endplates. The lower mitochondrial volume of type II fibers could render them for example more susceptible to death via mitochondrial dysfunction after oxidative damages[153]. However, such a mechanism is not suitable for the transgenic situation, since there is no indication that neurotrypsin overexpression and enhanced agrin cleavage at the NMJ directly influence the stability of the muscle fibers. Neither does greater stability of type I motoneurons apply for the transgenic situation, since the number of motoneurons is not changed at all. Most likely, differences in NMJ stabilities induce differences in the fiber type composition in conditions with enhanced agrin cleavage. Since the NMJ has been identified as primary target of neurotrypsin overexpression and since deterioration of the NMJ may induce the muscle degeneration, susceptibility of the NMJ to agrin-mediated stabilization or neurotrypsin-induced dispersal could decide the fate of the corresponding muscle fiber. Whether a similar mechanism is also responsible for the age-associated relative increase in type I fibers is not known, but the striking similarities in the phenotypes of aged and transgenic animals and the greater stability of

type I endplates to age-associated fragmentation are clearly favoring this hypothesis. Alternatively, fiber type distribution appears to be influenced by the expression of myogenic regulatory factors (MRFs), such as myogenin[611]. Myogenin expression was, for example, maximally detected in slow-twitch muscles. Age-related increase in myogenin expression may contribute to the type I fiber preference seen in sarcopenia. However, myogenin expression is not changed in neurotrypsin transgenic animals, despite a similar type I fiber preference. Therefore, increased myogenin expression may not be an essential condition for the age-associated increase in type I fibers. The same accounts for decreasing testosterone concentrations, which are thought to influence the fiber type distribution (see above). A continuously high androgen level appears to be required to maintain the low proportion of type I fibers in male compared to female mice[258]. Whereas an age-associated decrease in testosterone concentration has been found[259-261], such a decrease is unlikely to occur in young adult neurotrypsin-overexpressing animals, and hence, may not be an essential condition for type I fiber preference in sarcopenia.
Interestingly, in aged animals, we found that grouping is more pronounced in type II than in type I fibers, reflected by the higher T2 than T1 value. It's not completely clear whether this is indication of a biological process or just an artefact of the quantification method. The increase in type I fibers would rather suggest that reinnervation and reprogramming is more frequently accomplished by type I than by type II motoneurons. More frequent reinnervation of neighboring fibers then would result in more frequent grouping of type I and not type II fibers. Alternatively, if higher stability of type I NMJs in the aging process is assumed, denervation of type II fiber is more frequent. This process increases not only the amount of denervated type II fibers, but also the amount of unconnected type II motoneurons, which therefore could exhibit a higher potential for reinnervation. There might be reinnervation and reprogramming of type I fibers by type II motoneurons, although these connections might not be sustained for long time. Lexell and Downham[267] studied age-associated type grouping in 24 human vastus lateralis muscle and found a similar pattern in T1 and T2 values. Muscles from subjects around 30 years of age exhibited slightly negative values for both type I and type II fibers, nicely corresponding to our result for young adult wild-type mice. Thereafter, the cubic regression line for T2 slowly increased between 30 and 70 years of age, whereas T1 stayed constant until about 65 years of age. Thus, in muscles from subjects between 50 and 70 years of age, T2 was suspected to be greater than T1. The same pattern was observed in our study in mice in an analogical age range. However, a direct comparison of T1 and T2 values of the 24 individuals in the human study revealed a close agreement of T1 and T2. Since we analyzed only two time points, the age-related change in T1 and T2 cannot be fully understood. Furthermore, it is important to keep in mind that, unlike for type I fibers, several subgroups of type II fibers exist. Therefore, a group of type II fiber may actually consist of type IIa, IIx, or IIb fibers, which are innervated by different motoneurons. Quantification of type II fiber grouping should be made separately for each subgroup to more thoroughly analyze this issue. However, some of these fiber types may not or only in small numbers be present in the soleus muscle, making the quantification impossible.

4.11 Reliability of the method of enclosed fibers as indication of fiber type grouping

We used a method based on the number of enclosed fibers to analyze the degree of fiber type grouping. The concept of enclosed fiber has been introduced by Jennekens et al.[262] in order to collect data on the number of fibers in clusters. A muscle fiber was considered to be enclosed, if in a cross-section, it was completely surrounded by fibers of its own histochemical type. Most fibers are bordered by 4 or 5 other fibers, thus at least 5 or 6 fibers of one type have to occur together

to observe an enclosed fiber. Within large clusters the number of enclosed fibers is higher and the number of surrounding fibers is relatively lower than within small clusters. In general, fiber-type grouping is associated with an increase in the number of enclosed fibers. However, the number of enclosed fibers strongly depends on the relative amount of the fiber types. Thus, this method only leads to reliable results, if the proportion of the different fiber types stays constant. In our study, this was not the case. A relative increase in one fiber type necessarily increases the amount of enclosed fibers and a correction is needed. To study the statistical properties of the number of enclosed fibers, a model based upon hexagonal-shaped fibers was formulated[265]. In an empirical study, Downham et al.[655] showed that the number of enclosed fibers closely follows a negative binomial distribution, entirely defined by the sample size and fiber type proportion. Knowledge of the underlying distribution for a single sample now permitted the calculation of a significance level and the formation of a measure of randomness. However, the applicability of the model depends upon how close its assumptions resemble the muscle sections studied. In particular, there are two aspects that could invalidate the results. First, one fiber type could be consistently differing in size from the other type, and second the proportion of the two fiber types could systematically vary within a muscle. It is known that the different fiber types can vary considerably in thickness according to numerous factors including muscle type, species studied, and age of the subjects. In the present study, we did not observe a consistent difference in CSA between the fiber types in the soleus muscle of young wild-type animals. Neurotrypsin overexpression and aging increased the heterogeneity of fiber size, but not in a type specific way. Hypertrophied and atrophied fibers could be found among both type I and type II fibers. Without a consistent difference in thickness of one fiber type, the model of enclosed fiber should be valid, despite the increased heterogeneity of fiber size. A systematic variation in the proportion of fibers within the muscle was observed along the boarders of the soleus muscle in our study. Type II fibers were much more frequent along the boarder of the muscle than type I fibers. However, Lexell and Downham[267] formulated an alternative equation, which included a correction for the accumulation of one fiber type at the boarder of fascicles. This formula was used for the calculation of T values in this study. A further problem of the enclosed fiber method occurs, if one fiber type is much more frequent. In such a case, few, if any, enclosed fibers of one type can be expected, and so this count is unsuitable. Thus, the proportion of one fiber type should lie between 30 and 70% to generate reliable results. In this study, the fiber composition was in a reasonable range to use this quantification method. In most of the animals, the proportion of type I fibers was between 40 and 60%. There was only one case exceeding 70% of type I fibers and only three with more than 65%.

A major concern in our study was the high variation between individual animals in regard to T1 and T2 and the low correlation between T1 and T2 of the same animal. T1 for example varied between 2.2 and -1.2 for adult wild-type animals and a low T1 value was not an indication for a low T2 value in the same muscle. Lexell and Downham[267], in contrast, found a close correlation between T1 and T2 values. However, they used the human vastus lateralis muscle, which contains much more fibers than the mouse soleus muscle we analyzed. They assessed 18-60 fascicles with 700-2000 muscle fibers per individual, whereas we were bound to the 900-1000 muscle fibers of the mouse soleus muscle. Thus, the T1 and T2 value per individual may be more accurate in the human study. The low accuracy of the individual T values could explain the absence of a clear correlation between T1 and T2 in the same muscle. To compensate for the variation between the individuals we used high number of animals in each age group.

Together, the results that were obtained with this method were reasonable. Nevertheless, variations between animals, between muscles from both legs, and even between different sections from the same muscle were considerable, which is reflected by the high standard deviations. Thus, high numbers of cross-sections have to be used to get significant differences and small differences may

not be registered.

4.12 A model for neurotrypsin induced muscle wasting: the sequence of events

The overexpression of neurotrypsin in motoneurons leads to an excessive agrin cleavage at the NMJ. A 90 kDa middle fragment and a 22 kDa C-terminal fragment are produced. The C-terminal fragment of agrin, comprising the AChR aggregation activity, is diffusible and may leave the NMJ. The remaining agrin N-terminal part and the 90 kDa middle fragment, which might still be fixed at the basal lamina of the NMJ, are not capable to induce agrin signaling and protect the NMJ from dispersal. The presence of the nerve, releasing the dispersal factor acetylcholine, together with the absence of the protecting function of agrin leads to the loss of postsynaptic AChR aggregates. This results in dispersal of the presynaptic parts that are not directly opposed by postsynaptic sites. During this process, partial misalignment of the pre- and postsynaptic sites can be observed, although the alignment of pre- and postsynaptic sites is maintained in the majority of cases. The NMJs appear more and more fragmented. Eventually, the entire postsynapse of an NMJ is lost. Dispersal of all postsynaptic sites then is followed by the deterioration of the abandoned presynaptic nerve ending, which is not opposed to postsynaptic specialization and not supported by trophic factors from the muscle. The motoneuron itself is not affected and retains the ability for growth and innervation of other muscle fibers. Due to different stabilities, the majority of dispersed NMJs are of type II, leading to a higher proportion of type I endplates and consequentially type I fibers. The denervated muscle fiber is not supported by trophic factors and starts to atrophy, being angular and eventually to degenerate and get lost. The time a muscle fiber persists in a denervated state may be relatively short. A denervated muscle fiber can also be reinnervated by terminal sprouts of an adjacent motoneuron. However, in most of the cases this is not or only partially successful. Transient, unstable synapses are built rather than stable NMJs, which crucially depend on full-length agrin. Nevertheless, the takeover of denervated muscle fibers by sprouts from adjacent motoneurons, eventually leading to a type change, takes place, finally resulting in fiber type grouping. Whether it needs the formation of a complete NMJ or just some synaptic transmission is not clear. The potential of type I fibers to built endplates, regardless if stable or not, is higher, leading to a further increase in type I fibers. Agrin may play an important role in such reinnervation events, since additional supply of motoneurons with agrin increases their capability for synapse formation and takeover of fibers. Although some takeover of denervated fibers is possible, most of the muscle fibers, which looses their connection to the nerve are atrophied and subsequently lost. The loss of muscle fibers induces a regeneration reaction of the muscle. Satellite cells are activated and new, centrally nucleated fibers are added to the muscle. However, the innervation of these muscle fibers may not be successful in most of the cases, due to the lack of full-length agrin to form stable NMJs. Thus, the regeneration is ineffective and can't counteract the loss of fibers. Another compensation mechanism is more successful. Normal, non-fragmented endplates, capable of supporting myofibers, still exists in neurotrypsin-overexpressing animals, although their proportion is decreased. The healthy, correctly innervated fibers increase in thickness to compensate for the loss and atrophy of fibers. This is most likely a training effect, due to increased loading of the remaining fibers. Atrophy of denervated and compensatory hypertrophy of innervated fibers results in increased heterogeneity of fiber thickness. This process is highly dynamic; healthy endplates supporting hypertrophied fibers may also start to fragment and disappear, leading to atrophy of the corresponding fiber. Alternatively an atrophied fiber may become reinnervated and, when eventually a stable NMJ can be formed, hypertrophied. Due to this compensatory hypertrophy, the loss of muscle size and mass is relatively moderate, compared to the loss of muscle fibers. The reduction in muscle size leads to a loss of muscle strength of the

same magnitude. The de- and regenerating neuromuscular system complicates the control of the skeletal muscles and result in motor coordination impairment.
The pathogenic mechanism underlying sarcopenia may be similar. Destabilization of the NMJ could be the start point to a final common pathway controlling the pathogenic processes resulting in sarcopenia. Whether the mechanism inducing NMJ destabilization in senescence is also the same remains an open question.

4.13 Neurotrypsin-overexpressing mice can serve as model for sarcopenia

There are striking similarities between the loss of muscle mass and function due to neurotrypsin overexpression and due to aging. Beside the more general hallmarks of muscle weakness - reduced muscle strength and size, and loss of muscle fibers – features, that are special for sarcopenia - greater heterogeneity of fiber thickness, centralized nuclei, angular fibers, fiber type grouping and the increase in type I fiber proportion - can be observed in both skeletal muscle from aged and neurotrypsin-overexpressing mice. Moreover and most importantly, the fragmentation and decay of the NMJs that may initiate the process resulting in sarcopenia occurs in both aged and neurotrypsin transgenic animals. Thus, the neurotrypsin-overexpressing mouse can serve as an animal model for sarcopenia. The transgenic neurotrypsin-overexpressing mouse lines exhibit aging-like neuromuscular features in a well-defined, easy-to-handle fashion, which could simplify the analysis of aging induced degenerations dramatically. Research on sarcopenia bears a lot of drawbacks that could be circumvented using the neurotrypsin-overexpressing model. First, it is time consuming and expensive to generate aged mice, and experiments have to be planed a long time in advance. Second, the severity of sarcopenia in aged mice can vary substantially, increasing the amount of animals necessary to get reliable and consistent data. Especially mice from inbred strains often develop a variety of pathological alterations in senescence that cannot always be recognized and may influence the results. Third, the morphological alterations due to sarcopenia are rather small in mice, again increasing the amount of animals needed. It could be difficult to detect subtle changes for example upon treatment. Fourth, a variety of age-dependent changes that may or may not influence sarcopenia make it difficult to distinguish between modulating influences and fundamental mechanisms that underlie sarcopenia.
The neurotrypsin-overexpressing model provides an aging-like neuromuscular environment, in a consistent, well-defined way, and can be generated without extensive breeding time. Sarcopenia can be analyzed in young, otherwise healthy animals that do not exhibit other age-related changes, such as physical inactivity, reduced food uptake or endocrine alterations. A variety of influences that may modulate but not initially cause sarcopenia can be sorted out. Consequently, animals from the same mouse line exhibit a degree of sacropenia that is much more consistent than in naturally aged animals. Moreover, different mouse lines with distinct neurotrypsin expression levels provide a broad range of severities. Some transgenic mouse lines exhibit a phenotype, which is by far stronger than the naturally occurring. Thus, beneficial effects of a treatment could be detected much easier.

4.14 The neurotrypsin-overexpressing model provides insights into the pathogenesis of sarcopenia: NMJ stability is essential

Several hypotheses about the etiology of sarcopenia can be posted from the transgenic model: First, the deterioration of the NMJ is the essential factor triggering sarcopenia. Thereby agrin, as NMJ protecting and stabilizing agent, could play an important role. Second, a loss of motoneurons

does not precede the loss of fibers and may not primarily cause sarcopenia. Interestingly, fiber type grouping seen in sarcopenia does not necessarily indicate motoneuronal death, as it has been thought up to know. Instead, it may rather point to degeneration of the NMJs. Third, the potential of aged motoneurons to innervate denervated muscle fibers may be decreased due to the inability to form stable NMJs, which depend on agrin. The supply with full-length agrin might determine the success of reinnervation. And fourth, the preferential type II fiber loss is the result of a distinct type I and type II NMJ stability rather than the susceptibility of type I or II fibers to aging-induced processes[152]. Such processes, like the mitochondrial dysfunction, which arises from the accumulation, over time, of mitochondrial DNA damage as a result of oxidative stress, are not very likely to occur in neurotrypsin-overexpressing animals.
Factors influencing the stability of the NMJ can play a critical role in the development of sarcopenia. The structure of the NMJ has to be adjusted on the fine line between having room for plasticity and being stable over a long time period. Together, our results suggest agrin cleavage, NMJ destabilization, and fiber denervation at the onset of a final common pathway leading to sarcopenia. The interplay between agrin and neurotrypsin greatly influences the NMJ structure, making them candidates for an involvement in the process leading to sarcopenia.

4.15 The absence of neurotrypsin and the overexpression of a neurotrypsin-resistant agrin variant do not prevent sarcopenia

The induction of sarcopenia by overexpression of neurotrypsin in motoneurons prompted us to test for a role of enhanced neurotrypsin-dependent agrin cleavage as a pathogenic mechanism of age-dependent sarcopenia. We found that aged neurotrypsin-deficient mice suffered from sarcopenia as much as their wild type littermates. This indicates that neurotrypsin may not play an essential role in the pathogenesis of sarcopenia. However, the cleavage of agrin at the NMJ, and not the presence of neurotrypsin may be the important sarcopenia promoting process. Neurotrypsin overexpression could be regarded as a tool to induce extensive agrin cleavage at the NMJ, without actually playing a physiological role at this particular site. Recent results from our laboratory revealed that the overexpression of neurotrypsin-resistant agrin together with neurotrypsin results in a substantial delay, albeit not complete arrest, of NMJ maturation (Bolliger et al., in preparation). Because NMJ maturation was not delayed in Ntd mice we speculated that a distinct, but related protease, cleaving agrin at the same and possibly additional sites substitutes neurotrypsin as an agrin-cleaving protease at the NMJ. The hypothesized protease should not be expressed in the central nervous system due to the absence of cleavage fragments in Ntd mice. Such a protease might not exhibit as stringent site specificity as neurotrypsin. Neurotrypsin requires a highly restricted consensus sequence, with an acidic amino acid followed by a basic amino acid. Variances in P1 or P2 position completely abolish cleavage. Furthermore, neurotrypsin activity strongly depends on exosite interactions. Small peptide substrates with the consensus sequence are not cleaved. Consequently, only one substrate for neurotrypsin has been identified up to now. Most of the proteases are by far less specific, cleaving at multiple positions in a series of substrates. Therefore, it may well be that the proposed protease could process the neurotrypsin-resistant agrin variant, possibly with a decreased rate. In agreement with this hypothesis, the endplate maturation was delayed upon overexpression of neurotrypsin and resistant agrin but not completely prevented. The NMJs reached the pretzel stadium in Nto1/rAg animals, indicating that a low level of agrin cleavage might occur. The mutation of agrin at the neurotrypsin cleavage sites could reduce the rate of cleavage by the alternative protease without preventing it completely. The availability of less cleavage sites or less preferred cleavage sites is likely to reduce the rate of proteolytic processing. Alternatively, another agrin degrading process that is slower than the

cleavage by neurotrypsin or a related protease might exist. This process might ensure a basal level of agrin degradation and hence, NMJ maturation in Nto1/rAg animals. In accordance with these hypotheses, we found here that co-overexpression of a neurotrypsin-resistant form of agrin with neurotrypsin did not prevent sarcopenia. In contrast, Nto1/rAg double transgenic animals appear to suffer slightly more from sarcopenia. The neurotrypsin-resistant agrin might be cleaved or degraded by neurotrypsin independent processes over the animal's lifetime, leading to the destabilization of the NMJs that promotes sarcopenia.

Alternatively to increased agrin cleavage, agrin expression might be reduced with age. Deposition of a lower amount of agrin in the NMJ could have a similar effect as increased agrin cleavage, as both result in a lesser availability of agrin's signaling component. Indeed, Western blotting on aged spinal cord extract indicated, that the abundance of agrin might be lower in age. The upper part of the agrin signal, representing the most heavily glycanated variants, were absent in aged animals. Neurotrypsin can process these agrin variants, but might not or not alone be responsible for their absence in age, since they also disappeared in Ntd animals. In brain tissue, these variants have been shown to predominate at synapses. A lower amount of these, regardless whether due to increased degradation or decreased synthesis, could influence the stability of the NMJ and the development of sarcopenia. However, increased transgenic expression of agrin under the Thy-1 promoter (Ag and rAg mice) did not have a significant sarcopenia-preventing effect. Nevertheless, minor changes of several histological parameters have been noted. The age-associated loss of fibers appears to be less severe in senescent agrin-overexpressing animals than in wild type littermates. Consequentially, the compensatory hypertrophy was less pronounced in these animals. Since hypertrophy is induced by the loss of muscle fibers, it is not surprising that these two morphological parameters correlate. Most striking, the number of centrally nucleated fibers was significantly reduced in aged agrin-overexpressing animals compared to their wild type littermates. This could have several reasons: A reduced loss of muscle fibers may induce the compensatory addition of new fibers to a lower extent. However, aged agrin-overexpressing animals also lose a substantial amount of their muscle fibers in comparison to young animals. Therfore, it is not very likely that the regeneration mechanism is not induced to the maximal extent. Alternatively, in some skeletal muscles, hypertrophy is accompanied by an increase in centralized nuclei, most likely due to the fusion of satellite cells to the myofibers[656]. Since compensatory hypertrophy is less pronounced in Ag- and rAg-overexpressing than in wild type muscles, the amount of fused satellite cells and consequentially, centralized nuclei could be lower in these muscles. However, central nucleation was not especially frequent in hypertrophied fibers, suggesting that hypertrophy is not accompanied by central nucleation in the soleus muscle. Finally, the beneficial influence of transgenic agrin could be attributable to increased regeneration and not to the prevention of fundamental sarcopenia-inducing processes. Because in young animals overexpressing of wild type agrin induces fiber type switching and, by inference, reinnervation, an increased rate of reinnervation could be responsible for the beneficial effects of trangenic agrin in senescence. Centralized nuclei are thought to be evidence of newly formed fibers and disappear after innervation of the fibers. Increased reinnervation of newly formed fibers in aged Ag and rAg transgenic animals may reduce the amount of centrally nucleated fibers. In agreement with this hypothesis, a tendency for increased proportion of type I fibers was observed in aged agrin-overexpressing animals, suggesting that fiber type switching may be more frequent in these animals. Agrin might increase the possibility of a motoneuron to innervate denervated or newly formed muscle fibers. Newly built connections might only transiently be stable, but such a process could increase fiber type switching. In aged animals, the agrin degrading process might be slower than in neurotrypsin-overexpressing animals, and agrin overexpression could have a small beneficial effect on fiber number due to increased reinnervation, without actually counteracting

the fundamental process causing sarcopenia.
Importantly, the beneficial effects of agrin were observed upon overexpression of both wild type and cleavage-resistant agrin. This finding suggests that the additional supply with agrin does not counteract or delay an agrin degrading process that involves cleavage at the neurotrypsin –specific sites. If there is a sarcopenia-promoting decrease in full-length agrin, it might not result from increased cleavage at the neurotrypsin-specific position.
Together, two processes could result in decreased amounts of full-length agrin and, hence, in destabilization of NMJs and the propagation of sarcopenia. First, agrin could be locally cleaved at the NMJ by a protease with a proteolytic spectrum including but not limited to the α and β cleavage sites. Over an animal's lifetime, resistant agrin can't counteract the continuous degradation, which finally results in sarcopenia. Beneficial influence of transgenic agrin might rather indicate increased reinnervation of denervated or newly formed fibers than counteraction to the fundamental sarcopenia promoting process. Differences in cleavage rates between wild type and resistant agrin might be too small to observe a difference in the reinnervation-promoting effect. Second, decreased amounts of full-length agrin could be attributable to either reduced agrin synthesis or an agrin degradation process that is independent of cleavage at the α and β cleavage sites. This hypothesis would explain the beneficial effects of the overexpression of both neurotrypsin-resistant and wild type agrin. Since these effects are very small, agrin overexpression might not be sufficient to counteract the age-associated reduction. This would imply, either a low level of overexpression or a subsantial decrease in full-length agrin.
However, the evidence for an involvement of agrin cleavage in the pathogenesis of sarcopenia is rather weak. Other components of the NMJ could influence its stability. Age-dependent downregulation or decay of these components could similarly induce NMJ dispersal and activate a common pathway leading to sarcopenia. Oxidative damages due to age-associated increase in production of ROS could for example induce the degradation of stabilizing proteins. It has been shown that increased generation of ROS in superoxide dismutase (sod1)-deficient mice leads to fragmentation and denervation of the NMJs finally resulting in muscle fiber atrophy[460].

4.16 Could agrin be involved in age-related excitation-contraction uncoupling?

Another possibility of an influence of agrin cleavage on sarcopenia comes from the involvement of agrin in excitation-contraction coupling (ECC). It has been recently shown that neural agrin controls the maturation of the ECC mechanism in human myotubes *in vitro*[116]. Treatment of myotubes with agrin mimicks the effect of spinal cord explants in regard to the fraction of muscle cells showing a functional excitation-contraction mechanism. The promotion of ECC by agrin treatment involves the increase of functional ryanodine receptors and dihydropyridine-sensitive L-type Ca^{2+} channels. It has been shown that aging is associated with excitation-contraction uncoupling (ECU), which is mediated by an age-related decrease in expression of ryanodine receptors and dihydropyridine-sensitive L-type Ca^{2+} channels[155,156]. Enhanced agrin cleavage might potentially inactivate its function in controlling ECC. However, at the moment, there is no further evidence for such a connection. It is for example not known, which part of agrin is responsible for the control of ECC. Furthermore, agrin's effect on ECC has only been found in human and not in mouse muscle cells, whereas age-associated ECU has been found in mice. Further experiments would be needed to examine a possible role of agrin in age-related ECU.

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Curriculum Vitae

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Publications

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